Journal of
DAIRY SCIENCE

Vol. 44 September, 1961 Number 9

CONTENTS

PEOPLE AND EVENTS 3

STUDENT CHAPTER NEWS 9

GENERAL INTEREST:

Pesticide Residues and the Dairy Industry.
H. E. O. Heineman and C. B. Miller 1775

Publication Problems of the American Dairy Science Association.
E. L. Jack 1782

Can the Journal of Dairy Science Be Useful to Latin American Countries?
Earl Weaver 1783

Call for Papers for the 1962 Annual Meeting of the American Dairy Science Association.
M. E. Senger 1784

Status of the Journal of Dairy Science for 1960.
E. O. Herreid 1785

RESEARCH PAPERS:

Lipid Components of the Fat-Globule Membrane.
M. P. Thompson, J. R. Brunner, C. M. Stine, and Karin Lindquist . 1589

Migration of Phosphatides in Processing Dairy Products.
George R. Greenbank and Michael J. Pallansch 1597

Effects of Direct Steam Heating and Vacuum Treatments on the Chemical Composition of Milk with Especial Reference to Substances Involved in Oxidized Flavor Development.
D. H. Kleyn and W. F. Shipe, Jr. 1603

Crystalline Rennin in the Manufacture of Cheddar Cheese.
P. M. Linklater and C. A. Ernstrom 1621

Modified Reduction Test to Predict the Shelf Life of Cottage Cheese.
L. G. Harmon, G. C. Walker, and L. D. McGilliard 1627

Method for Determination of Citric and Lactic Acids in Dairy Products.
D. R. Frazeur 1638

Factors Related to the Flavor Stability of Foam-Dried Whole Milk. I. Effect of Oxygen Level.
A. Tamsma, M. J. Pallansch, T. J. Mucha, and W. I. Patterson . . 1644

Chemical State and Nutritional Availability of Iron in a Prepared Infant Formula.
O. W. Vaughan and R. E. Knauff 1650

Influence of the Stage of Lactation and Environmental Temperatures on the Salt Balance of Milk.
T. H. Kamal, H. D. Johnson, and A. C. Ragsdale 1655

Reproductive Capacity of Dairy Bulls. II. Gonadal and Extra-Gonadal Sperm Reserves as Determined by Direct Counts and Depletion Trials; Dimensions and Weight of Genitalia.
J. O. Almquist and R. P. Amann 1668

(Continued on inside front cover)

CONTENTS

(Continued from front cover)

Acid-Soluble Phosphorus Constituents of Bull Semen in Relation to Fructolysis and to Sperm Concentration.
M. H. Ehlers, Joan Dixon, and R. E. Erb 1679

Source of Endogenous Pyruvic Acid in Bovine Seminal Fluid, and Utilization.
William G. R. Marden 1688

Female and Breed Associated with Seasonal Fertility Variation in Dairy Cattle.
G. H. Stott 1698

Measurement of Genetic Trend.
L. D. VanVleck and C. R. Henderson 1705

Milk Replacers for Dairy Calves. I. A Comparison of an All Milk By-Product Replacer and Limited Whole Milk With and Without Chlortetracycline.
F. R. Murdock, A. S. Hodgson, and T. H. Blosser 1711

Changes in Body Weight During the First Pregnancy for Holstein Heifers Calving at Different Seasons.
A. B. Schultze and H. P. Davis 1717

Growth of the Calf Udder Using DNA as an Index.
Ralph Williams and C. W. Turner 1721

Udder Capacity Estimates as Affected by Rate of Milk Secretion and Intramammary Pressure.
H. A. Tucker, R. P. Reece, and R. E. Mather 1725

Method for Increased Milk Production with Rotational Grazing.
H. T. Bryant, R. E. Blaser, R. C. Hammes, Jr., and W. A. Hardison . 1733

Comparison of Continuous and Rotational Grazing of Three Forage Mixtures by Dairy Cows.
H. T. Bryant, R. E. Blaser, R. C. Hammes, Jr., and W. A. Hardison . 1742

Thyroid Supplementation at Peak of Lactation.
H. A. Tucker and R. P. Reece 1751

Metabolism of Radiostrontium in Young Pigs and in Lactating Rats Fed Stable Strontium.
John C. Bartley and Elwood F. Reber 1754

TECHNICAL NOTES:

β-Lactoglobulin and pH-Sensitivity of the Heat Stability of Evaporated Milk.
Dyson Rose 1763

Observations on the Free Volatile Acids in Milk.
W. J. Harper, I. A. Gould, and C. L. Hankinson 1764

Furacin Residues in Milk.
R. W. Henningson 1765

Tracheal Tube for the Total Collection of Rumen and Respiratory Gases.
D. R. Waldo and H. Hoernicke 1766

Volatile Fatty Acid Analyses of Blood and Rumen Fluid by Gas Chromatography.
E. S. Erwin, G. J. Marco, and E. M. Emery 1768

Experimental Production of Bloat with Various Chemicals.
E. C. Leffel and R. J. Komarek 1771

Growth Stimulation of *Butyrivibrio* by Mucin.
G. R. Gordon and W. E. C. Moore 1772

Palmitaldehydrogenic Substance in Bovine Sperm Lipids.
Stuart Patton and Robert J. Flipse 1773

OUR INDUSTRY TODAY:

See title under General Interest.

ASSOCIATION AFFAIRS:

See titles under General Interest.

Price Schedule for Reprints of Papers That Appear in the Journal of Dairy Science.
H. F. Judkins 1789

JOURNAL OF DAIRY SCIENCE

OFFICIAL PUBLICATION OF THE
AMERICAN DAIRY SCIENCE ASSOCIATION

E. O. HERREID, Editor-in-Chief, University of Illinois
Editorial Address: Station A, Box 718, Champaign, Illinois
Phone: EMpire 7-7073

EDITORIAL BOARD

C. K. JOHNS, Ottawa, Canada
H. D. EATON, Storrs, Conn.
R. E. ELY, Reno, Nevada
F. A. KUMMEROW, Urbana, Ill.
N. L. JACOBSON, Ames, Iowa
ROBERT JENNESS, St. Paul, Minn.
J. J. JEZESKI, St. Paul, Minn.
J. E. LEGATES, Raleigh, N. C.
E. P. REINEKE, East Lansing, Mich.
DURWARD OLDS, Lexington, Ky.
STUART PATTON, University Park, Pa.
M. L. SPECK, Raleigh, N. C.
E. L. JACK, Davis, Calif.
N. L. VANDEMARK, Urbana, Ill.
EVERT WALLENFELDT, Madison, Wis.
G. M. WARD, Manhattan, Kan.
U. S. ASHWORTH, Pullman, Wash.
C. A. ZITTLE, Philadelphia, Pa.
R. W. TOUCHBERRY, Urbana, Ill.

COMMITTEE ON JOURNAL MANAGEMENT

J. F. CAVANAUGH, Chairman
American Jersey Cattle Club, 1521 East Broad Street, Columbus, Ohio

H. A. HERMAN
F. J. DOAN
E. O. HERREID, ex officio
H. F. JUDKINS, ex officio

OFFICERS OF THE ASSOCIATION

E. L. JACK, President
Department of Food Science and Technology, University of California, Davis, Calif.

I. W. RUPEL, Vice-President
Department of Dairy Science, Texas A & M College, College Station, Texas

H. F. JUDKINS, Sec.-Treas.
32 Ridgeway Circle, White Plains, N. Y.

E. O. HERREID, Journal Editor-in-Chief
Champaign, Ill.

DIRECTORS

S. T. COULTER, St. Paul, Minn.
F. J. DOAN, University Park, Pa.
R. E. HODGSON, Beltsville, Md.
RAYMOND ALBRECTSEN, Ithaca, N.Y.
J. T. REID, Ithaca, N. Y.
C. W. NIBLER, Lincoln, Nebr.
G. H. WISE, Raleigh, N. C.
E. O. HERREID, ex officio
H. F. JUDKINS, ex officio

ADMINISTRATIVE INFORMATION

Manuscripts

Manuscripts for publication in the Journal of Dairy Science should be submitted to the editor, E. O. Herreid, at the address above. See January, 1954, issue, page 103, for information concerning the preparation of manuscripts, or send for reprint, "Information for Authors."

Advertising

Advertising plates, copy, insertion orders, contracts, and requests for information relating to advertising in the Journal should be addressed to the Journal of Dairy Science in care of The Garrard Press, 510–522 North Hickory Street, Champaign, Illinois.

Nonmember Subscriptions

Nonmember subscriptions are $15.00 per volume in the United States and Canada; $15.50 in all other countries, net and postpaid. New subscriptions and renewals begin with the first issue of the current volume. Renewals should be sent in by December 1 of the current year for the following calendar year to avoid a break in the series. Subscriptions should be sent to the Journal of Dairy Science in care of The Garrard Press, 510–522 North Hickory Street, Champaign, Illinois.

Subscriptions for the British Isles and the British Empire should be ordered through our agents: Messrs. Bailliere, Tindall and Cox, 7 and 8 Henrietta Street, Covent Garden, London, W.C. 2, England. Subscriptions for Australia should be sent to our agent: John H. Bryant, Herbert Street, St. Leonards, N.S.W., Australia.

Changes of mailing addresses, post office notices of undeliverable copies, inquiries about copies lost in the mail, and all other matters pertaining to the mailing list for nonmember subscribers should be sent to The Journal of Dairy Science in care of The Garrard Press at the above address. Claims for copies of the Journal of Dairy Science lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

Memberships

Membership dues in the Association are $10.00 a year and should be sent to H. F. Judkins, Secretary-Treasurer, 32 Ridgeway Circle, White Plains, New York. Renewals should be made not later than December 1 each year.

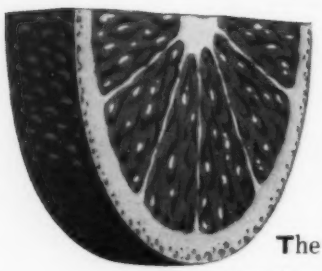
Changes of mailing addresses, inquiries about copies lost in the mail, requests for back copies and all other matters pertaining to the mailing list for members of the American Dairy Science Association should be sent to Mr. Judkins at the above address. Claims for copies of the Journal of Dairy Science lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

Form 3579 to be returned to Garrard Press

Printed by The Garrard Press, 510–522 North Hickory Street, Champaign, Illinois. Entered as second-class matter at the post office at Champaign, Illinois, under the act of March 3, 1879.

Copyright, 1961, by the American Dairy Science Association. Printed in the U. S. A.

THIS IMPORTANT SLICE OF THE DAIRY BUSINESS CALLS FOR KRAFT QUALITY



The fruit bases that dairies buy to make orange, lemon and grape drinks *must be* of uniform freshness and quality—so it's no wonder that the swing is to fruit bases by Kraft. All are made under the careful supervision of Kraft's food experts and backed by Kraft's reputation for the finest food products.

Kraft Orange Base, for example, is produced from lush tree-ripened oranges. And the drink it makes will have a true fresh-squeezed flavor. Always just right, always uniform.

To those dairies who use Kraft fruit bases, Kraft dependability means fruit drinks that equal, in quality and uniformity, their own fine milk products. Would *you* like samples for classroom work? Just write your nearest Kraft office.

KRAFT FOODS DAIRY SERVICE DIVISION
500 Peshtigo Ct., Chicago 90, Illinois • New York • Garland, Texas • San Francisco

KRAFT

PEOPLE AND EVENTS

Virgil O. Dreyer Killed in London Accident

V. O. Dreyer, of Dayton, Ohio, was struck by a motorcycle May 23 in London, England, traffic, and passed away May 25. He and Mrs. Dreyer had been scheduled to sail from Southampton, England, on the last leg of a six-weeks' European tour. He had retired as Manager of the Borden Company's Dayton milk division December 31, 1959.

Dreyer was born in Ross County, five miles north of Chillicothe, Ohio, December 20, 1894. He attended grade school in a one-room brick schoolhouse two miles from home. Using a bicycle or a horse and buggy, he graduated from Chillicothe High School in 1913. He was graduated from the Department of Dairying, College of Agriculture, Ohio State University, in 1917. He was a member of the 1916 Perfect-Record Buckeye Squad and earned a letter as Ohio State's Most Faithful Substitute. He was also a member of the Alpha Zeta Agricultural fraternity.

In 1917 Dreyer joined the Navy and was assigned to the Brooklyn Navy Yard, where he helped equip a new hospital ship, the U.S.S. Comfort. This ship was closely identified with the Atlantic fleet in the transfer home of casualties from the Western front. After a few months on this ship, he was detached and sent to Princeton University for three months' intensive training in Officer School for the Supply Corps. Upon being commissioned an Ensign Paymaster, he was transferred to the Fifth Naval District in San Francisco. He married Maria Kraft Swope in March, 1918, at Lancaster, Ohio. Following her death, he married Irma Louise Decker, in 1926.

Following his graduation from Ohio State he secured employment with the Dairy Products Company. He worked in the laboratory and plant processing department, securing experience in actual practice and applying the theories learned in college. After his honorable discharge from the Navy, he resumed employment with the Dairy Products Company and worked his way up to Production Manager. In 1930 the company became affiliated with The Borden Company, at which time he was given the post of Assistant Treasurer. In 1941, Borden's Dairy Products Company and Borden's Finch Jersey farms were merged, with Dreyer as manager.

Among the many honors Dreyer achieved were the following: In 1943 he was appointed a member of a seven-man advisory committee of the War Food Administration. He was elected a member of The American Jersey Cattle Club in March, 1944. He received recognition from The Dairy Industry Committee, Washington, D. C., in August, 1945, for the leadership displayed as state industry chairman for Ohio, in executing the 1945 eight-point dairy program.

During World War II he trained with Auxiliary Police and at night rode with the Ohio State Patrol. He received a Certificate of Distinguished Service from the Ohio State Patrol in April, 1947.

He was also Vice-President of the American Dairy Association, in 1947 becoming a member of their Board of Directors.

He was a member of the Dayton Chamber of Commerce and served on various committees: a member of the Horace A. Irvin No. 647 F. & A.M.; Victory Chapter No. 210 Royal Arch Masons, Reese Council No. 9 Royal and Select Masons; Reed Commandry No. 6 Knight Templars and Antioch Shrine Temple A.A.-O.N.M.S. During 1931 he was Commander of Reed Commandry No. 6, and in 1939 was High Priest of Royal Arch Masons, Victory Chapter.

One of his most cherished awards was given to him by the Department of Dairy Technology, Ohio State University in 1956, in recognition of his outstanding contributions to the dairy industry during the preceding 39 years. In that same year, the Honor Society of Agriculture, Gamma Sigma Delta, presented Dreyer with a Certificate of Membership, in recognition of his scholarship and his service to Agricultural Service.

Among other honors: Dreyer received a testimonial from the Ohio Dairy Products Association for more than 40 years of faithful and devoted service to the Dairy Industry; also, they at one time gave him the presidency. The Dayton Milk Foundation also presented him a tribute in sincere appreciation of his many years of devoted service to Dairy Industry. He was president of this association at one time. He was a member of Borden's Quarter Century Club, having completed 42 years of continuous service. In February 9, 1960, he was elected a Life Member of the American Dairy Science Association.

BUT DOCTOR, WHY DO I NEED MORE CALCIUM NOW?

The time to prepare for the physiological demands of motherhood is in the formative adolescent years.

Nowadays, the trend to earlier marriages makes it even more desirable that full calcification of the skeleton be achieved at an early age. Complete calcification of the bones by this age requires an average retention of about 400 mg. of calcium daily during most of adolescence. For girls 13 to 19 years old a calcium intake of 1.4 grams per day is recommended . . . along with 400 I.U. of vitamin D. Only with a liberal consumption of milk and milk products . . . at least the equivalent of 4 glasses daily . . . is calcium retention likely to reach this objective.

The expectant mother should receive extra calcium during the second half of pregnancy . . . or earlier if her dietary history is poor. An allowance of 1.5 grams daily is recommended . . . to be followed by a further increase to 2.0 grams during lactation. As three-fourths of the calcium in the national food supply comes from milk and milk products . . . low consumption of these foods usually reduces the calcium below recommended allowances. If weight control is a problem and certain food restrictions are recommended, proper food selection must be further emphasized . . . to insure adequate calcium intake, *plus* adequate protein, vitamin and other nutrient consumption. Milk products . . . of a wide variety . . . can be utilized with ease in weight control programs.

Milk and other dairy foods stand alone in their ability to provide the calcium needed by the pregnant and lactating mother in a form that is highly palatable, easily digested, and readily absorbed . . . and they also provide other nutrients which contribute to a balanced dietary and buoyant health.

The nutritional statements made in this advertisement have been reviewed by the Council on Foods and Nutrition of the American Medical Association and found consistent with current authoritative medical opinion.

Since 1915 . . . promoting health through nutrition research and education

NATIONAL DAIRY COUNCIL

A non-profit organization

111 NORTH CANAL STREET • CHICAGO 6, ILLINOIS

AVAILABLE ON REQUEST:
Reprints of this series of messages on Calcium
NEW CALORIE-RESTRICTED DIET SHEETS

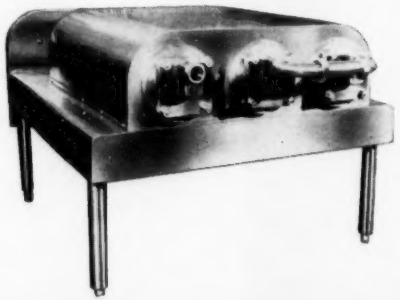
heat exchangers

fit your needs with top efficiency

THE *Creamery Package*® MFG. COMPANY

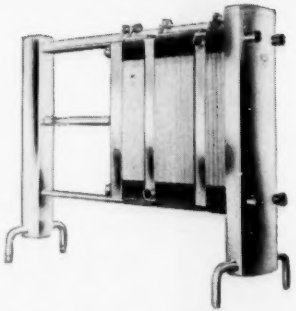
General and Export Offices:
1243 W. Washington Blvd., Chicago 7, Ill.
Branches in 24 Principal Cities

These TWO, of the quality-built CP Heat Exchangers will fill many of YOUR processing requirements:



Swept Surface® HEAT EXCHANGER

designed for continuously heating or cooling products which tend to form surface films on other heat exchangers and for heating and cooling to extremes of temperature. Dasher blades, sweeping over the heat exchange surface, actively prevent burn-on and increase heat exchange by elimination of filming. Models are available for slush freezing, chilling, cooling, heating and cooking. Efficient, durable, low maintenance—CUTS YOUR PROCESSING COSTS. Ask for bulletin E-1300.

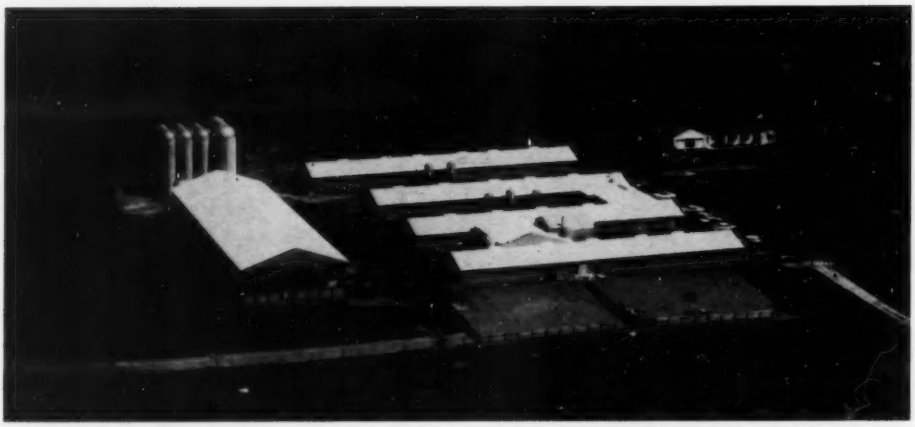


CRESCENT® HEAT EXCHANGER

to heat or cool fluid products. Plates lend themselves to efficient regenerative heating and cooling — recovers heat — saves refrigeration. Plates have *exclusive* CP Unimold gaskets—integrally bonded to the plate — cementing is eliminated. Only every-other-plate is gasketed—only half as many gaskets—yet every plate is an effective heat exchanger. Design is rugged, sanitary, functional, for years of trouble-free, low cost processing. Write for bulletin E-1153.

UNIVERSITY NEWS

New Dairy Cattle Research and Teaching Center, Michigan State University



At left: The pole-type hay barn. A small-batch hay dryer for special trials is installed in the south end. At north end: Six silos 10 by 40 ft. and two 10 by 40 ft. Constructed to collect, measure, analyze seepage from each silo. The four long barns—extending east and west—stanchions, poles as support, aluminum siding and roof, gutter cleaners, cement-asbestos board as interior finish. First barn (nearest camera)—80 cows, stanchion pipeline. Between first and second barn—feed storage, milk room with two bulk tanks. Second barn—34 cows, portable pipeline, office, facilities. Between second and third barns—laboratory, metabolism stalls. Third barn—calves, stalls for fistula animals, classroom, restraining stanchion. Elevated metal bins for feed storage are visible. Fourth barn—heifers. Looking north, one-half mile, with the two silos: the remodeled loose housing unit for the Brown Swiss and Jersey herds; all herd sires housed here. Farther north, one and one-half miles: some buildings on the campus. Toward east: The swine center with the University feed mixing plant.

The new Dairy Cattle Research and Teaching Center, Michigan State University, was formally dedicated September 1, 1961, with farmers, dairy breeders, and other visitors from over the state in attendance.

On arrival, visitors were first conducted on a tour of the new Center. With C. A. Lassiter, Department Head, as chairman, the program was presented under two over-all topics. Under the topic Dedicated to Serve, R. M. Swenson, Assistant Dean, discussed Teaching: L. M. Turk, Station Director, Research; and N. P. Ralston, Extension Director, Extension. Frank Merriman, Board of Trustees, represented that body. T. K. Cowden, Dean, had the assignment, Michigan State University's Responsibility to Michigan Agriculture.

Under the other over-all topic, Research on Parade, Donald Hillman, Extension Specialist, presented a paper, Effect of Soybean Oil Meal on Mastitis and Sterility; L. D. Brown, Research Instructor, presented, Results of High Level Grain Feeding; R. S. Emery, Associate Professor, Cause and Prevention of Fat Test Depression; H. D. Hafs, Research Assistant Professor, Progress Report of Sex Control Studies in Dairy Cattle; J. W. Thomas, Professor, Results of Feeding Hydroponic Forage.

Cattle were moved into the new Center on July 19, 1961. The old dairy barn, experimental barn, and bull barn, all located too close to encroaching new dormitories and other buildings of the main campus, are entirely vacated and are being razed.

North Dakota

P. W. Aschbacher Granted Leave

DR. P. W. ASCHBACHER has been granted a leave of absence for one year from North Dakota State University to continue his studies at the Atomic Energy Commission Agricultural Research Laboratory at the University of Tennessee. He will study goiter development in calves, using radio-tracer techniques.

Ohio

The 13th Annual Homecoming Brunch conducted by the Department of Dairy Technology, The Ohio State University, will be at 10 A.M., November 4, in the Auditorium of The Agricultural Administration Building. Each year this popular affair affords an opportunity for alumni of the Department to renew old friendships and relationships with classmates and staff members, and to become

aware of recent Departmental activities. In addition to the period of informal socializing immediately preceding the Brunch, special recognition of the classes of '11, '21, '31 '41, and '51 will be made. Presentation of The 1961 Award of Merit of the Department of Dairy Technology, as well as the Department's Certificate of Appreciation to an outstanding member of the Ohio Dairy Industry, will be made during the program.

Two staff members of the Department of Dairy Technology, The Ohio State University, participated in the 34th Annual Dairy Day held by the Ohio Agricultural Experiment Station at Wooster on August 11. DR. M. F. GREGORY, Extension Specialist, presented a discussion entitled The ABC of Milk Sanitation and Quality. As part of a special program for women in attendance, D. D. COLE discussed the properties and uses of various cheese in his presentation The World of Cheese.

DR. W. J. HARPER is recuperating from severe leg burns suffered on July 17 when a container of hot coffee overturned while he and his family were visiting friends. Five weeks of hospitalization and several weeks of limited activity resulted from the accident. Just prior to his hospitalization, Dr. Harper's contributions to the Department of Dairy Technology, were given further recognition when the Board of Trustees of The Ohio State University announced his promotion to the rank of full Professor.

DR. C. V. MORR has joined the staff of the Department of Dairy Technology, The Ohio State University, and will conduct research on the effect of heat treatment on the milk proteins system under a grant from the National Institute of Health. Dr. Morr was on the staff of the Research Laboratories of the Carnation Company prior to assuming his new position at Ohio State.

Staff members and graduate students of the Department of Dairy Technology, The Ohio State University, enjoyed a weekend outing at Indian Lake, near Bellefontaine, Ohio, August 4-6. The cottage and boating facilities of MR. FRANK WISH, of Hopewell Dairy in Bellefontaine, were placed at the Department's disposal for this enjoyable affair.

DR. K. S. RAMACHANDRAN has left the staff of the Department of Dairy Technology at Ohio State to return to his native India. While with the department he conducted research on the chemical nature of chocolate ice cream flavor under a grant from The American Dairy Association. He will take an extensive tour through Europe and the Middle East on his homeward journey.

MAKE YOUR CHOCOLATE DRINK A ALE LEADER

Start with **American Food's** Chocolate Drink Concentrate! This fully prepared **Dairy Powder** contains all the necessary ingredients for making a delicious chocolate milk drink of the **best quality** possible. Made only of the **finest cocoas**, **American Food's** concentrate results in a chocolate milk drink with a taste-tempting color that's so important to **sales appeal**. And its "chocolatey" **flavor** and uniform body will keep your product sold all year round. You'll realize production **savings**, too, with this simple and economical product.

American Food's Chocolate Drink Concentrate comes in single or extra strength in **pre-weighed** bags for any size batch. Your first batch will convince you — There's no **finer quality** in chocolate milk drinks.

Write to our **Technical Service** Staff for complete information.

CARE AND CONTROL ★
AMERICAN FOOD LABORATORIES, INC.
1000 STANLEY AVENUE, BROOKLYN 8, N. Y.
1442 FLOWER STREET, GLENDALE 1, CALIFORNIA
PHILADELPHIA · BALTIMORE · DETROIT · CHICAGO · DALLAS

HOW TO MAKE THE WORLD'S BEST ICE CREAM

You must use the finest dairy ingredients, the finest flavors, the finest mix processing and freezing equipment, the finest packaging—*And The Finest Stabilizer*—KELCO STABILIZER.

Dariloid XL® *for your white mixes*
Sherbelizer® *for your chocolate mixes*
Products of **KELCO COMPANY**

75 Terminal Ave., Clark, N. J.
20 N. Wacker Drive, Chicago 6, Ill.
530 W. Sixth St., Los Angeles 14, Calif.
Cable Address: KELCOALGIN—CLARKNEWJERSEY

Accurate profit-control is the job of Mojonnier Milk Testers for standardizing butterfat and total solids content. • Mojonnier Testers have been a must for profitable dairy operation over 45 years. • Fat Determination accurate to .03 of 1%. Total Solids Tests accurate to 1/10th of 1%.

Write for full details today

MOJONNIER BROS. CO.
4601 W. OHIO ST., CHICAGO 44, ILLINOIS

Mojonnier MILK TESTERS
QUALITY ENGINEERED FOR ECONOMY

STANDARD THROUGHOUT THE WORLD

D. D. Cole resigned his position with the Department of Dairy Technology, The Ohio State University, effective August 11, to take a position in the home office of The Creamery Package Corporation in Chicago.

Mr. Owen Latimer, a chemical engineer with the New Zealand Dairy Research Institute, recently visited the Department of Dairy Technology, The Ohio State University, where he discussed with several staff members the Department's research on the effects of heat on concentrated and dried milk products. Mr. Latimer will conduct research in this area on his return to New Zealand following a number of similar visits to various academic and industrial research organizations.

Tennessee

Wylie Returns to Illinois

Professor Emeritus C. E. Wylie will return to Southern Illinois University in September for the school year. He will be Visiting Professor in the Animal Industries Department, teaching courses in nutrition and advanced dairy feeding.

Professor Wylie was on the faculty of the University of Tennessee for 44 years. He retired as Head of the Dairy Department last August. During the spring quarter, 1961, he assisted in developing and teaching a new dairy course for graduate students at S.I.U. Mrs. Wylie will accompany Prof. Wylie to S.I.U.

U. of Tennessee Dairy Club Honors A. M. Glover

The 1961 Dairy Service Award by the University of Tennessee Dairy Club was presented to A. M. Glover at the Club's annual banquet. This was the first year of the award, which will be made annually. The award consists of a citation and a framed portrait of the honoree which will be placed in McCord Hall, home of the Department of Dairying on the University of Tennessee campus. Mr. Glover, long-time manager of the Knoxville Milk Producers Association, was selected because of his many years of service in that position as well as other important Dairy Industry activities. These include leadership in the organization of an Artificial Breeding Cooperative, The Dairy Council of Knoxville, The American Dairy Association of Tennessee, Milk Marketing Organizations, Milk Quality work, and many more activities all of which have enhanced the welfare of the dairy farmers and the dairy industry in Tennessee.

A. M. Glover, right, receiving the first Dairy Service Award from Guy Walker, left, who read the citation at the University of Tennessee Dairy Club's annual banquet.

STUDENT NEWS SECTION

J. E. JOHNSTON, Editor

A Section Devoted to News of Student Members

Student Branch of the A.D.S.A. Annual Meeting June 11-14, 1961

Launching the National Student Branch, like launching our first astronaut, required that many people devote their skills and services. The success of the meeting at Madison is indeed a gratifying tribute to all who have made this launch possible.

Two luncheons were held, which enabled over 50 student affiliates to meet with more than 20 outstanding men of the Association. Through close contact fostered by eating together, many young leaders were able to gain from the experiences of our senior members. This year, as last year, it was acclaimed a most worthwhile function.

In the general meetings many items were discussed and a number of motions passed. The major motions approved by the delegates that will affect all members are:

(1) To cover the necessary operating expenses of the National Student Branch, each affiliated chapter will be charged $5 annually, payable to the Secretary-Treasurer on or before the date the annual reports are due.

(2) The membership adopted an emblem. President Lineweaver had designed, obtained approval, and investigated the cost of using it in various ways as part of the wearing apparel.

(3) Due to cost considerations, the discussion of the emblem was tabled until next year; however, it was suggested that the emblem be incorporated into a lapel pin or tie pin.

Undoubtedly, our most successful and enlightening function at the Annual Meeting was the symposium, Selecting a Career in the

FISKE MILK CRYOSCOPES
Available for
IMMEDIATE DELIVERY

STOP! ADDED WATER IN MILK

FISKE MILK CRYOSCOPES determine accurate water content in milk and other dairy products by the freezing-point method. An aid to dairies and processors in controlling the quality of their product. Enables public health authorities and testing laboratories to process test samples rapidly and easily with minimum of technically trained personnel.

Today's FISKE MILK CRYOSCOPES, available in laboratory or portable models, offer these . . .

NEW, EXCLUSIVE FEATURES:

- SHOCK FREEZING
- DUAL STIRRING RODS
- BUILT-IN INTERVAL TIMER
- STAINLESS STEEL UNBREAKABLE TEMPERATURE PROBE
- INTEGRATED PRE-CHILL BATH

for complete details, specifications and applications, write to:

FISKE ASSOCIATES, INC.
BETHEL, CONNECTICUT
Creators of
Precision Electronic Devices

For the finest
Chocolate ice cream in town
quality

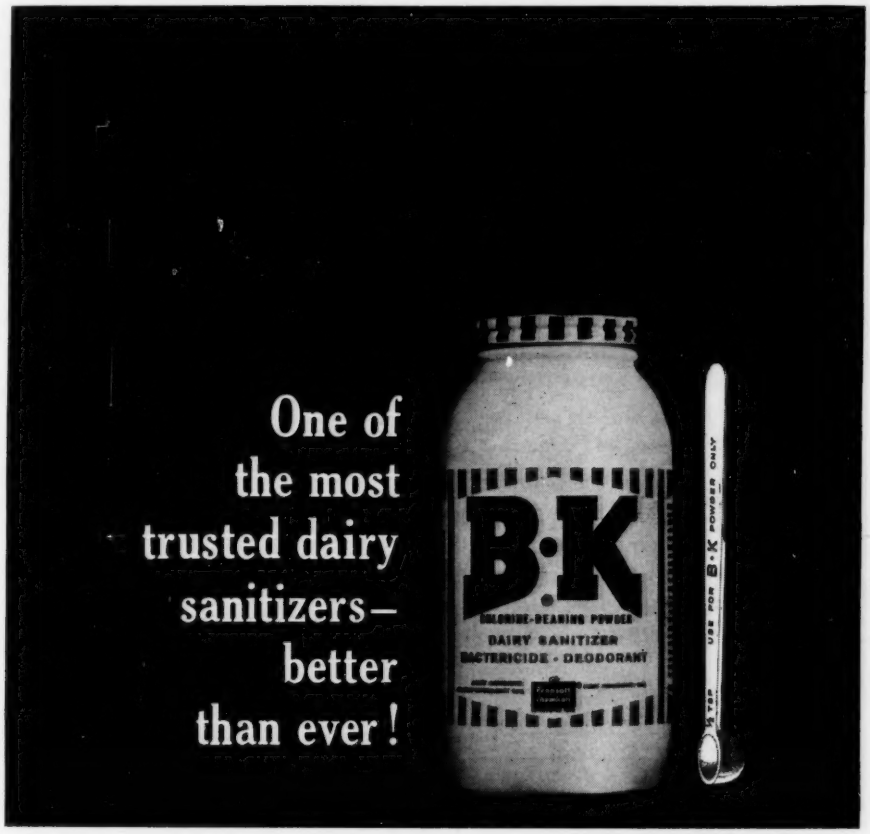
Cocoas and
Chocolate flavors

You, too, can have "the finest Chocolate ice cream in town" when you use Ambrosia's distinctive Dutch Process Cocoas, Pure Chocolate Liquors, or Chocolate Flavors. Why not try Ambrosia for your Chocolate Ice Cream?

Ambrosia
FOOD OF THE GODS

For samples and more information write to:
AMBROSIA CHOCOLATE COMPANY
Milwaukee 3, Wisconsin

Manufacturers of fine chocolate and cocoa since 1894



. . . and in a new unbreakable container

New, improved B-K Powder has greater advantages than ever: • Economical • Dependable • In new lightweight red, blue and white polyethylene container—safe, unbreakable • No more problem of broken glass in milkhouse • Free plastic measuring spoon inside container.

Another product for healthful living from
PENNSALT CHEMICALS CORPORATION
B-K Department
East: 3 Penn Center, Philadelphia 2, Pa.
West: 2700 S. Eastern Ave., Los Angeles 22, Calif.

Pennsalt Chemicals®
ESTABLISHED 1850

WRITE FOR FREE CULTURE BOOKLET

Eliminate lost batches, save time, speed production. Use *Numbered Blends* and the Dairy Laboratories system of rotation that supplies highest quality fresh culture *every week* to keep aroma, body, flavor and production uniform in all fermented milk products.

FLAV-O-LAC FLAKES

Write for details in our free Culture Booklet

DALARE ASSOCIATES

THE DAIRY LABORATORIES
2300 Locust Street
Philadelphia 3, Pennsylvania

Branches: New York • Washington, D.C.

MARSCHALL

complete
LAB SERVICE
for the
DAIRY
and
FOOD INDUSTRY

Highly specialized technologists put Marschall's 50 years of experience to work on your quality and production control problems.

- bacterial quality tests
- antibiotic tests
- bacteriophage tests
- "staph" presence tests
- proteolytic tests on pasteurized milk, cream and butter
- pesticide and insecticide tests
- all other quality control tests

MARSCHALL DAIRY LABORATORY, Inc.
TESTING DIVISION
P. O. Box 592 • Madison 1, Wisconsin

Dairy Industry. Thanks to the wisdom and experience of each participant, we all learned of new opportunities in education and research, foreign service, production, and manufacturing.

Congratulations are in order to the Georgia Chapter for winning the 1961 Outstanding Student Affiliate Chapter Award. There were many excellent annual publications displayed this year; however, you will note that the judges' score-card for evaluating the total activities of the club lists other important points which involve the work of the entire club and not just the reporters.

The new officers, selected to guide the National Student Branch during the coming year are:

Joseph Lineweaver, President, Virginia
Jacob Megehee, First Vice-President, Mississippi
Keith Jenkins, Second Vice-President, Ohio
Dennis Boesiger, Third Vice-President, Nebraska
Camilla Cristman, Secretary-Treasurer, Michigan

At the close of the meetings, the newly elected and the retiring officers closely examined this year's activities and laid plans for next year's program at Maryland.

CAMILLA CRISTMAN
Secretary-Treasurer,

Annual Meeting of American Public Health Association

More than 5,000 public health specialists from this country and abroad are expected to attend the 89th annual meeting of the American Public Health Association and meetings of about 60 related organizations at Cobo Hall in New York City November 13-17. Registration will be open to nonmembers.

Among subjects of major sessions will be medical care for the aged; evaluation of health education, including adult health education; dental radiological hygiene programs; dental needs of cerebral palsy children; accident prevention; air pollution; coordination of environmental health services in metropolitan areas; current activities in international health; swimming pool sanitation; significant developments in various aspects of environmental health, and the concept of disease eradication.

Also, cancer epidemiology, new developments in multiple antigen vaccines, live attenuated measles vaccine, coronary heart disease, staphylococcal infectious, respiratory viral infections, teenage nutrition, rehabilitation including vocational rehabilitation of the mentally ill, nursing home standards, cancer screening programs, and chemicals and food safety.

American Cottage Cheese Institute Annual Meeting in October

The American Cottage Cheese Institute announces a three-day Cottage Cheese Clinic to be held in conjunction with the Department of Dairy and Food Science, Cornell University, Ithaca, N. Y., October 18, 19, and 20.

Advances in Cottage cheese manufacture and distribution will be the high light of the session, with many discussion and demonstration periods devoted to production, quality control, and marketing of Cottage cheese. One afternoon will be devoted entirely to flavors in Cottage cheese, with nationally noted speakers covering all aspects of the subject. A field trip to local New York cheese operations is planned for the last day of the meeting.

INDUSTRY NEWS

Cherry-Burrell Opens New Distribution Center

Cherry-Burrell Corporation has just opened an additional sales and service center at Mountainside, New Jersey. The center will serve the Eastern United States with high-speed warehousing facilities.

The Mountainside (Eastern) Sales and Service Center will back up the existing sales branches in the Eastern United States with parts and supplies. Requests for parts and supplies are relayed by teletype to the Mountainside center from the sales branches. These messages are converted to orders by 100-word-per-minute electronic machines, and within minutes the parts or supplies are packaged and ready for shipment.

LABORATORY SERVICES

Applied Research and Development, Testing and Consultation

Food, Feed, Drug and Chemical Analyses, Animal Studies, Pesticide Screening, Pesticide and Additive Residue Analyses.

For price schedule and specific work proposals, write

WISCONSIN ALUMNI RESEARCH FOUNDATION

P. O. Box 2217, Madison 5, Wisconsin

DAIRYLAND
FOOD LABORATORIES, INC.

WAUKESHA WISCONSIN

DFL

Serving the Dairy Industry with Enzymes and Enzyme Modified Products

AMERICAN RENNET EXTRACT ■ DFL PEPSIN COAGULANT ■ ITALASE ■ CAPALASE ■ BLUE CHEESE FLAVOR ■ MIL-LAIT ■ LIPOLYZED BUTTER OIL EMULSION ■ CHEESE COLOR REFERENCE STANDARDS ■ TESTING PREPARATIONS AND EQUIPMENT

E. L. Byers Named Ice Cream Judge in Collegiate Dairy Products Judging Contest

A former fellowship winner in the Collegiate Students' International Contest in Judging Dairy Products will be the official ice cream judge when the contest is staged in Washington, D.C., October 23.

E. L. BYERS, Laboratory Specialist with the Bowman Dairy Company, Chicago, has been appointed official ice cream judge by Contest Superintendent L. H. BURGWALD, of the U.S. Department of Agriculture, Washington, D.C. He succeeds J. H. ERB of the Borden Company, Columbus, Ohio, who is retiring after 14 years of service as ice cream judge.

In October, 1934, Mr. Byers was a member of the Kansas State University's dairy products judging team which won the fellowship in the contest held that year in Cleveland, Ohio. He then entered the University of Wisconsin, from which he received the M.S. degree in 1936. For the next three years, he was plant manager of the University of Wisconsin Creamery and, in 1939, he joined Bowman Dairy Company, where he has remained for the past 22 years. The majority of his time is spent on quality control of ice cream.

FOR TECHNICAL HELP . . .

call the man from **Blumenthal**

In addition to supplying you with high quality chocolate flavorings and coatings for your ice cream, The Man From Blumenthal will gladly:

1. Discuss prices and prevailing market conditions.
2. Arrange for his laboratory to perform special services that can only be handled by a lab staffed with chocolate technicians and equipped with the latest analytical and pilot plant equipment to produce special types of chocolate for ice cream.
3. Refer your problems to one of our consultants specializing in technical ice cream problems.
4. Conduct research on new ideas suggested by you.

BLUMENTHAL BROS. CHOCOLATE CO.

MARGARET AND JAMES STS., PHILADELPHIA 37, PA.

Meet the World's Best Judges of Ice Cream . .

Your Customers*

Only your customers will determine whether your special ice cream flavor is a good selection and seller. Will they come back for more? Will they tell others how good it is? They alone, your customers, will judge the goodness and quality. Look for your answers in your sales analysis report.

*with due respect to your flavor panel

Competition is such today that, if you do not offer your customers what they want and like, they will buy elsewhere. You cannot afford to lose that business. To avoid disappointment and to insure a successful and profitable program, let us help you with the many selections we have to offer.

In the interest of the ice cream manufacturer we have developed the unique RAMSEY PLAN FOR MERCHANDISING ICE CREAM which offers you a guide and a wide selection for a successful and profitable program. Our contribution includes the presentation of ice cream samples for your ready-to-taste appraisal.

For full particulars write or call

RAMSEY LABORATORIES, INC.

2742 GRAND AVENUE CLEVELAND 14, OHIO SWeetbriar 1-2727

Mr. Byers joins four other distinguished judges of dairy products whose rulings on product samples are the basic criteria from which scores are judged in the annual contest. Other judges, and their product specialties, are: D. A. PETTEE, The Creamery Package Mfg. Company, Boston, Milk; ROBERT JOHNS, Kraft Foods Company, Chicago, Cheese; B. J. ALDRICH, H. C. Christians Company, Chicago, Butter; and N. E. FABRICIUS, Dairy Maid Products Cooperative, Eau Claire, Wis., All Products.

E. C. Haskell Promoted in Borden's

E. C. HASKELL has been named production manager of milk-based products for the Borden Foods Company. Since January, 1958, Mr. Haskell has been assistant production manager of milk-based products, which include Starlac nonfat dry milk, sweetened condensed and evaporated milks, Borden's Instant Double-Malted Malted Milk, and Borden's Instant Hemo, a vitamin-mineral fortified chocolate drink mix.

Mr. Haskell joined Borden's in 1931 as a clerk. He became office manager of the company's Union City, Pa., operations in 1932, assistant product manager at the firm's New York headquarters in 1935, assistant superintendent of Borden's Ft. Scott, Kansas plant in 1949, superintendent at Albany, Oregon, in 1951, and assistant division manager at Syracuse, New York, in 1953. He returned to New York in 1957 as an assistant product manager for milk-based products.

Drohan Named Foremost Dairies, Inc. Manager

T. E. DROHAN has been appointed product manager for milk and ice cream by Foremost Dairies, Inc.

Drohan, who is at Foremost's San Francisco headquarters, was formerly sales and marketing manager of the Manteca Canning Co. in San Francisco. Earlier, he had been a product manager for the Foods Division of Lever Brothers and the advertising manager of Best Foods, both in New York City. He resides in Tiburon.

Completed Thesis

M.S. Degree:

KOWDUR SUNDERRAM HEDGE—A study of the effects of feeding low level rates of phenothiazine and diethylstilbestrol to young dairy calves. The University of Georgia.

KLENZADE
Where Progress Keeps Ahead of Tomorrow
Klenzade
Expanding Technology
Aids Dairy Industry to Higher Quality Standards
MORE NEW PRODUCTS
New approaches, procedures, formulations, and packaging improvements.
MORE AUTOMATION
Expanded engineering staff for plant and farm automation and CIP cleaning.
MORE FARM SERVICES
Enlarged division for pipeline milker and bulk tank quality clinics and cleaning demonstrations.
MORE CULTURE SERVICES
Extended line of cultures, products, related equipment, in-plant problem assistance.
MORE EQUIPMENT
Wider range for plant and farm automation, spray sanitizing, acidified rinsing, proportional feeding.
MORE RESEARCH
Added facilities for product development, basic research, materials testing, trouble-shooting.
MORE MANPOWER
Ever-broadening field representation, sales-service, and technical assistance.
KLENZADE PRODUCTS, INC.
Systematized Sanitation All Over the Nation
DEPT. 20J BELOIT, WISCONSIN

FIGHT MASTITIS WITH A MILKER?

Yes! Gentle De Laval milking can help you do it!

It's no secret that rough milking machines, or improper milking, can predispose dairy cows to mastitis. That's why you should know there's a method of milking that's remarkably *gentle* on cows' sensitive teats and udder tissues . . . so gentle it can help you and dairymen in the fight to keep herds mastitis-free.

De Laval milking provides maximum protection to udders to help any mastitis prevention program work better. Here are the reasons why:

1. Other milkers use wide-bore liners that cause cows' teats to "balloon" to fill extra space. De Laval uses narrow-bores that fit snug, reduce impact when liners collapse, minimizing danger of udder injury.

2. Our magnetic pulsation assures *absolutely uniform milking* with never a variation. Pulsators are factory set, won't get out of adjustment.

3. De Laval cuts vacuum drop-offs that cause slow, erratic milking. Our *rotary* vacuum pump assures rapid vacuum recovery for faster, more uniform milking.

These exclusive features are the difference between harsh milking and the *gentle* milking dairymen need for maximum protection of cows' udders to help prevent mastitis. Gentle milking is built right into every De Laval milker . . . Suspended, Pail-Type, Cow-to-Can® and Combine Milker®. For further information, write: The De Laval Separator Co., Poughkeepsie, N. Y., or 5724 N. Pulaski, Chicago 46, Ill., or 201 E. Millbrae Ave., Millbrae, California.

pioneers in better milking for more than 75 years

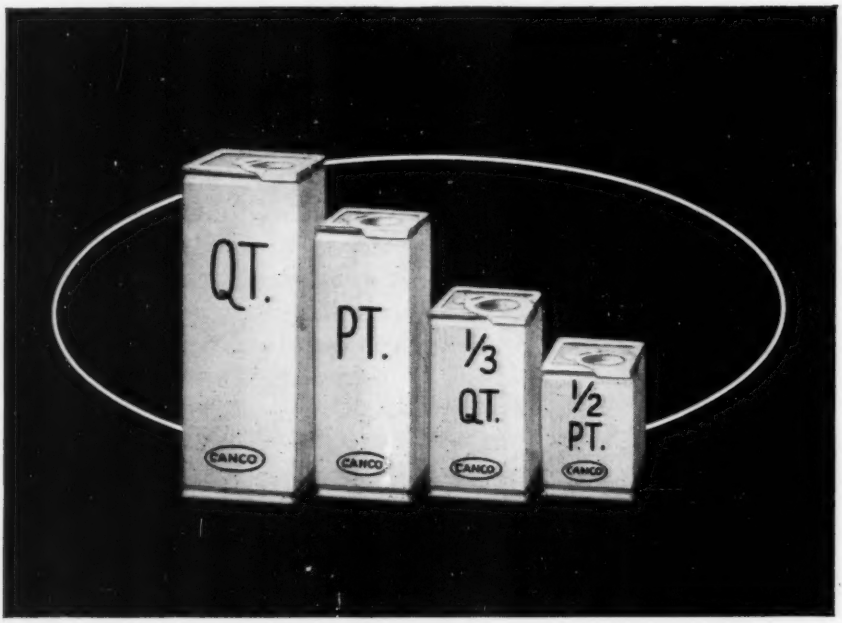
THE MAGIC TOUCH OF LACTOSE will boost per capita consumption of fluid milk products!

Research at the university and industry level has shown that Edible Lactose, the natural sugar of milk, gives fluid milk products a distinct flavor advantage. Result: realistic product improvements that consumers readily accept. **BUTTERMILK**—Lactose returns the "old fashioned" goodness, tangy aroma and mellow smoothness. It tones down acid sharpness without affecting body development. **SKIM MILK** (modified or fortified)—Lactose builds fuller body and a slightly sweeter taste. Chalky, stale and weak, uninteresting flavors are avoided. **CHOCOLATE DRINK**—Lactose contributes to a richer, more chocolaty taste, smoother body and longer stability. For complete details, get in touch with Western, world's largest producer of Lactose—pure milk sugar. Do it today!

Western Condensing Company • Appleton, Wisconsin

Division of Foremost Dairies, Inc.

foremost lactose



MILK

IN GRADE "A" CARTONS

Canco's paper containers, the original disposable cartons for milk, have been winning greater acceptance every year! Popular with homemakers everywhere, these containers are ideal for use in school cafeterias and vending machines. They are easy to open and close; provide "controlled pouring," and are compact, sturdy, sanitary. Yes, Canco cartons are the *preferred* containers for milk . . . the Grade "A" cartons for nature's most nearly perfect food!

CANCO Division AMERICAN CAN COMPANY

ADVERTISERS' INDEX

Ambrosia Chocolate Company 10
American Can Company 18
American Food Laboratories, Inc. 7
Australian Society of Dairy Technology 19
Blumenthal Bros. Chocolate Company 14
Creamery Package Mfg. Co. 5
Dairy Laboratories 12
Dairyland Food Laboratories, Inc. 13
De Laval Separator Co. 16
Difco Laboratories ..... Cover 4
Fiske Associates, Inc. 9
Kelco Manufacturing Co. 8
Klenzade Products, Inc. 15
Kraft Foods 2
Marschall Dairy Laboratory, Inc. 12
Mojonnier Bros. Company 8
National Dairy Council 4
Pennsalt Chemicals Corp. 11
Ramsey Laboratories, Inc. 14
Technical Industries, Inc. 20
Western Condensing Company 17
Wisconsin Alumni Research Foundation 13

The Australian Journal of Dairy Technology

The Journal contains scientific and technical papers on all aspects of dairying, especially dairy manufacture. It provides for those in other countries an up-to-the minute record of the latest developments in dairy technology in Australia and New Zealand. News, reports, editorials, book reviews and Society proceedings are included, and the Journal is illustrated. It appears quarterly.

SOME ARTICLES PUBLISHED IN 1960

Food-Borne Epidemics Due to Staphylococci, by E. Munch-Petersen
The Manufacture of Lactic Acid and Its Use as a Precipitant for Casein, by N. Oetiker
Estimation of Quantity of Fat in Bulk Cream, by F. H. McDowall
The Use of Brilliant Blue F.C.F. in Intramammary Penicillin Preparations, by D. J. Dawson and J. T. Feagan
An Improved Curd-Fusing Machine, by J. Czulak, N. H. Freeman and L. A. Hammond
Improved Equipment for the Manufacture of Casein, by L. L. Muller and J. F. Hayes
Obtainable through booksellers, or from the Australian Society of Dairy Technology, P. O. Box 20, Highett, Victoria, Australia. Price £ Stg. 1/14/0 per annum.

Special Offer: **A certain number of complete sets of back numbers, comprising 12 volumes and a decennial index, are available at a concession price of £ Stg. 10.**

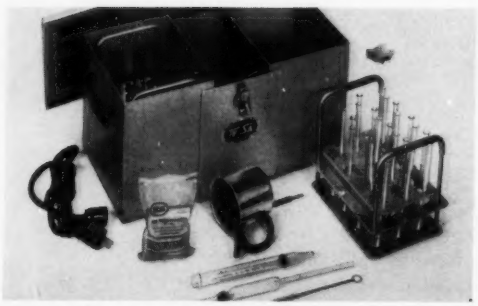
TECHNICAL INDUSTRIES, INC. TE SA FORT LAUDERDALE, FLA.

BUTTERFAT TEST METHOD

IS NOW ACCEPTED FOR ALL HERD PRODUCTION TESTS —

•

Advanced Registry Record of Performance
Register of Merit
HIR
DHIR
DHIA
Owner-sampler



No centrifuge required. No acid. Eliminates danger to eyes and clothing. Accuracy proven by university tests. The only simple, safe, accurate method of butterfat testing. The low cost practical method for farm or laboratory.

1959 Approved by ADSA Dairy Records Committee, subject to AOAC action.

1959 Adopted as first action by AOAC.

1960 Approved for raw and preserved samples by ADSA Dairy Records Committee.

1961 Approved by PDCA Records Committee.

1961 Approved by all dairy breeds.

See your dairy equipment dealer or write

TECHNICAL INDUSTRIES, INC.
2711 S.W. 2nd AVE., FORT LAUDERDALE, FLA.
Please send me further information
Name_____ T
Address_____
City_____ State_____ DSJ

RESEARCH PAPERS

LIPID COMPONENTS OF THE FAT-GLOBULE MEMBRANE [1]

M. P. THOMPSON,[2, 3] J. R. BRUNNER, C. M. STINE, AND KARIN LINDQUIST
Department of Food Science, Michigan State University, East Lansing

SUMMARY

Lipid components were extracted from fat-globule membranes prepared by two procedures. One method (Scheme I) started with cold, pooled milk, whereas in the other procedure (Scheme II) freshly drawn, uncooled milk served as the source. The lipids obtained were resolved by gradient elution from a silicic acid chromatographic column.

The following lipid classes were identified as components of the fat-globule membrane: carotenoids, a squalene-like compound, cholesterol esters, cholesterol, a unique triglyceride mixture similar to the high-melting glyceride fraction, mono-diglycerides, phospholipids and, from some preparations, unesterified fatty acids. The minor differences in composition between the membrane lipids reflected the age of the milk and the method of preparation. Membranes isolated from the pooled milk by Scheme I contained lower concentrations of phospholipids and associated proteins. Carotenoids, cholesterol esters, and the squalene-like component were removed from the membranes by washing with ethyl ether.

The lipid-protein nature of the fat-globule membrane has been recognized for years. Palmer (3, 7) and his associates partially characterized a high-melting glyceride fraction (HMG) which they obtained from the lipid fraction of the membrane. This fraction has been studied in more detail and reported to contain relatively large quantities of C_{14}, C_{16}, and C_{18} saturated fatty acids, in addition to some $C_{18}^{=}$ and traces of $C_{18}^{2=}$ and $C_{18}^{3=}$ (6, 9). The membrane lipids are not restricted to the HMGF. Phospholipids, unsaponifiable materials such as the carotenoids, vitamin A, and cholesterol and its esters have been identified as constituents of the membrane (1, 5, 10).

This paper serves to report characteristics of the total lipid fraction of the fat-globule membrane.

EXPERIMENTAL PROCEDURE

Fat membranes were isolated from washed, raw cream obtained from pooled milk delivered to the MSU Dairy Plant during the summer months. The isolation procedure is represented by Scheme I, Figure 1. The membranes prepared under these conditions were subjected to varying degrees of induced lipolysis resulting from variations in temperature and processing manipulations inherent in the collection of the milk and the preparative procedures. To minimize these influences, membranes were isolated from freshly drawn, uncooled milk according to the procedure outlined in Scheme II, Figure 1. The membrane-containing serum or washed-cream buttermilk thus obtained was washed two times with

Received for publication May 11, 1961.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2815.

[2] This paper is part of a dissertation presented to the Faculty of the College of Agriculture of Michigan State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Present address: Eastern Utilization Research Branch, USDA, Philadelphia, Pa.

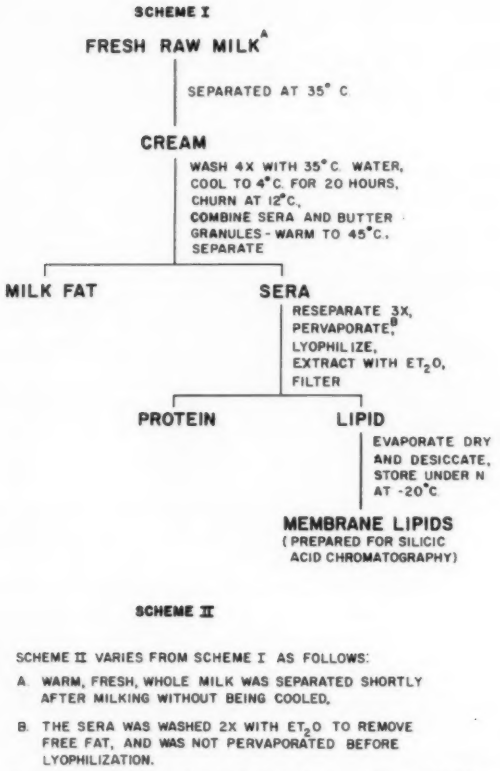

SCHEME I
FRESH RAW MILK[A]
SEPARATED AT 35° C
CREAM
WASH 4X WITH 35° C. WATER, COOL TO 4° C. FOR 20 HOURS, CHURN AT 12° C., COMBINE SERA AND BUTTER GRANULES - WARM TO 45° C., SEPARATE
MILK FAT
SERA
RESEPARATE 3X, PERVAPORATE,[B] LYOPHILIZE, EXTRACT WITH ET_2O, FILTER
PROTEIN
LIPID
EVAPORATE DRY AND DESICCATE, STORE UNDER N AT -20° C
MEMBRANE LIPIDS
(PREPARED FOR SILICIC ACID CHROMATOGRAPHY)
SCHEME II
SCHEME II VARIES FROM SCHEME I AS FOLLOWS:
A. WARM, FRESH, WHOLE MILK WAS SEPARATED SHORTLY AFTER MILKING WITHOUT BEING COOLED.
B. THE SERA WAS WASHED 2X WITH ET_2O TO REMOVE FREE FAT, AND WAS NOT PERVAPORATED BEFORE LYOPHILIZATION.

FIG. 1. Procedures for isolating fat membrane lipids.

twice its volume of ethyl ether, to remove the loosely bound or peripherally adsorbed lipids. Further, the serum was freeze-dried without prior concentration.

Lipids were extracted from the membrane preparations with a mixture of 35% ethanol in ethyl ether, followed by three washings with ethyl ether (Scheme I) or with ethyl ether alone (Scheme II). The extracted membranes consisted of proteinaceous material, containing only traces ($< 2\%$) of extractable lipids. Total lipid specimens, representing the native lipid of the milk used in these isolations, were extracted from a portion of the washed creams just prior to churning, by a solvent extraction scheme consisting of ammonium hydroxide, ethanol, ethyl ether, and petroleum ether—somewhat similar to the Mojonnier extraction technique.

Characterization of the extracted lipids was achieved by chromatographic resolution employing the essential features of the silicic acid[4] column technique described by Hirsch and Ahrens (2).

[4] Silicic acid (minus 325 mesh) was obtained from Bio-Rad Laboratories, Richmond, California.

A standard chromatogram was developed by chromatographing a mixture consisting of authentic samples of squalene, cholesteryl palmitate, α-tocopherol, tripalmitin, stearic acid, cholesterol, monoglycerides, and the phospholipids lecithin, cephalin, and sphingomyelin, Figure 2. Recovery of the initial charge of 214.2 mg. approximated 97%. Squalene, α-tocopherol, and cholesterol were not quantitatively recovered, reflecting either the impurity of the original compound or the incompleteness of their elution from the column. The presence of impurities in the authentic compounds was verified by characteristics of their infrared spectra. The remaining lipids were recovered quantitatively but with somewhat different elution characteristics than reported by Hirsch and Ahrens (2), a deviation attributed to the absence of nitrogen pressure on the column, but which appeared to be inconsequential in view of the reproducibility of the elution patterns in successive trials.

Identification of the various classes of lipids present in the various lipid fractions was made on the basis of their position of elution from the silicic acid

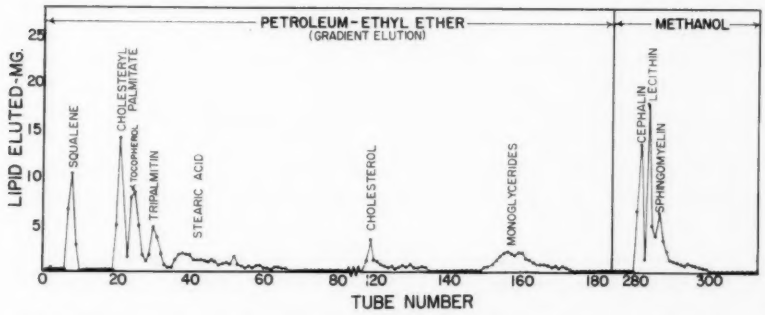


FIG. 2. Elution pattern of a mixture of authentic lipids from an 18-g. column of silicic acid. Lipid charge was 214.3 mg.

column and on their infrared spectral characteristics. For the latter characterization, a Beckman IR-5, double-beam spectrophotometer was employed. Approximately 5% solutions of the lipid, carried in spectrophotometric-grade CCl_4, were injected into a 0.2-ml. cell with a path length of 0.002 mm.

The HMG fraction and the membrane-triglyceride fraction were prepared for gas chromatographic analyses by methanolysis in Skellysolve A, according to a method employed by Smith and Jack (8). The resulting fatty-acid methyl-esters were resolved on a 10-ft. firebrick packed–column containing diethyleneglycol succinate (DEGS) as the liquid phase. Identification of the elution peaks was achieved by comparing their characteristics with those for authentic fatty-acid methyl-esters. The column was operated at approximately 180° C. in an Aerograph Model A-90-C apparatus equipped with a four-filament thermal conductivity cell. Injection temperature was maintained at approximately 250° C. with a H_e flow rate of about 75 ml/min. A 3-μl. sample was injected.

RESULTS AND DISCUSSION

Figure 3 shows a typical elution pattern for the total lipid fraction extracted from washed cream. The appearance of large quantities of triglyceride (Peak 3) complicates the observation of the eluted α-tocopherol. The carotenoids (Peak 1) and cholesterol (Peak 4) are indigenous to butteroil. The component(s) represented by Peak 2 remains unassigned, but was thought to be squalene or a squalene-like cholesterol intermediate. Small quantities of phospholipids appeared in the methanol eluate, but were not included in the chromatogram. The gross heterogeneity of the neutral triglyceride, as evidenced by the multiple-peaked elution area, was expected in view of the numerous combinations of mixed triglycerides that exist in milk fat. Mono- and/or diglycerides were not observed, possibly because of their relatively low concentration in the total fraction.

The chromatogram shown in Figures 4 and 5 represent typical elution patterns of membrane lipids prepared from Schemes I (471.4 mg. charge) and II (441.6 mg. charge), respectively. Reported membrane isolations showed characteristically similar chromatograms. Identification and yields of the components constituting the peak areas are presented in Tables 1 and 2, respectively. Similar elution patterns were obtained for smaller lipid charges ($\sim$ 250 mg.), but with better resolution of the component peak areas. The latter technique was not pursued, for the reason that yields of minor components were, in most cases, too small for accurate quantitative and qualitative determinations.

The chromatograms developed from the elution of these two membrane lipid preparations reflect, in all probability, the preparative procedures employed in

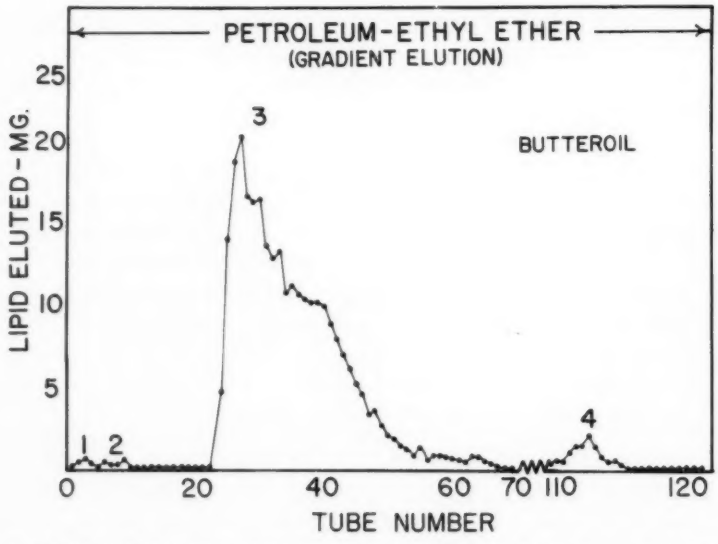


FIG. 3. Silicic acid chromatographic separation of milk fat. Lipid charge was 424.9 g.

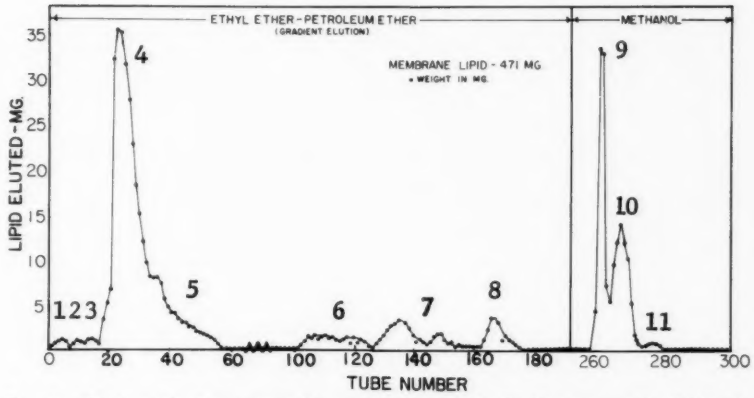


FIG. 4. Silicic acid chromatographic separation of the lipid components obtained from membranes prepared according to Scheme I, Figure 1. Lipid charge was 471.4 mg.

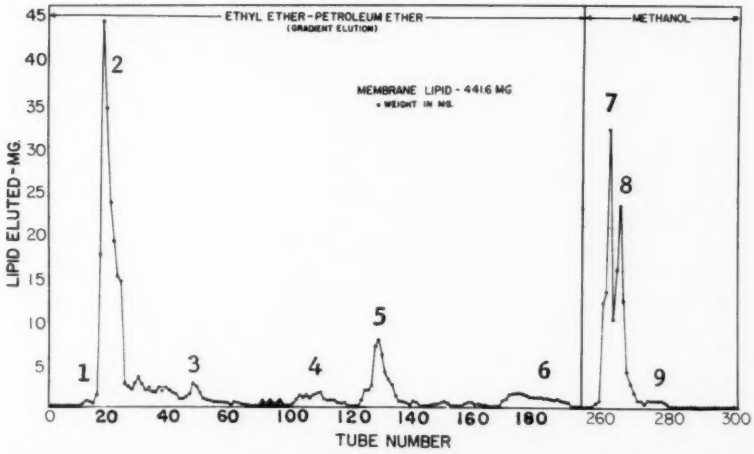


FIG. 5. The chromatographic separation of the lipid components obtained from membranes prepared according to Scheme II, Figure 1. Lipid charge was 441.6 mg.

their isolation. The chromatogram for the lipids of the Scheme I preparation showed approximately 6% of free fatty acids, whereas the lipids of the Scheme II preparation showed only traces. These results suggest that induced lipolysis was encountered at one or more steps in the isolation of membrane lipids by the procedure outlined in Scheme I. Quite likely, the pooled, raw milk used as the starting raw material for the preparation was the principal contributing factor. On the other hand, the total mono-diglycerides content of the preparation can not be attributed to lipolysis, since these components were observed in the membrane lipid fraction prepared by Scheme II. Conceivably, the mono-diglycerides,

TABLE 1

Composition of the lipid fraction of the fat-globule membrane prepared by Scheme I

Lipid [a]	Peak No.	Lipid eluted from silicic acid	Percentage of membrane lipids	Percentage of whole membrane [b]
		(mg.)	*(%)*	*(%)*
Carotenoids	1	2.2	0.45	0.30
Squalene [b]	2	3.0	0.61	0.40
Cholesterol esters	3	3.9	0.79	0.54
Triglycerides	4	263.0	53.41	36.12
Free fatty acids plus other triglycerides	5	31.0	6.30	4.26
Cholesterol	6	25.4	5.17	3.50
Diglycerides	7	40.0	8.14	5.49
Monoglycerides	8	22.9	4.66	3.14
Phospholipids	9, 10, 11	100.0	20.35	13.76
Totals		491.4	99.88	67.51
Percentage recovery		104.3*(%)*		

[a] Listed in order of elution from silicic acid column (Figure 4).
[b] Membrane consisted of 32.49% protein.
[c] Precise identification pending.

TABLE 2

Composition of the lipid fraction of the fat-globule membrane prepared by Scheme II

Lipid [a]	Peak No.	Lipid eluted from silicic acid	Percentage of membrane lipids	Percentage of whole membrane [b]
		(mg.)	*(%)*	*(%)*
Squalene [c]	....	0	0	0
Carotenoids	....	0	0	0
Cholesterol esters	1	2.9	0.63	.27
Triglycerides	2	230.9	49.98	21.88
Free fatty acids plus other triglycerides	3	Traces	.....	.....
Cholesterol	4	16.8	3.64	1.59
Diglycerides	5	48.9	10.58	4.63
Monoglycerides	6	29.8	6.45	2.82
Phospholipids	7, 8, 9	132.7	28.72	12.57
Totals		462.0	100.00	43.76
Per cent recovery		104.6		

[a] Listed in order of elution from silicic acid column (Figure 5).
[b] Membrane consisted of 56.24% protein.
[c] Precise identification pending.

although present in relatively small concentrations in milk fat (4), are selectively oriented at the fat-plasma interface, thus constitutng a normal component of the membrane lpids. Their presence may account, in part, for the physical stability of the fat emulsion.

Washing the fat membranes with ethyl ether resulted in the removal of the loosely bound carotenoids, cholesterol, and the squalene-like compound. The cholesterol esters were not affected by this treatment, an observation suggesting that these lipids are more tenaciously oriented into the membrane structure. Further, Scheme II preparations contained relatively larger quantities of phospholipids in their lipid phase and more total protein than found in Scheme I preparations. The cooling and agitation of commercially collected, pooled milk, along with the subsequent manipulations of the isolation procedure encountered

in Scheme I, may have resulted in the desorption of phospholipids, proteins, and/or lipoproteins.

A comparison of the chromatograms for the membrane lipids (Figures 4 and 5) and total milk lipids (Figure 3) revealed some differences in the resolution of the elution patterns. The relatively high concentration of broad-spectrum triglycerides encountered in the milk lipid specimen appeared to mask several of the minor lipid components. In contrast, the triglyceride peaks appearing in the membrane lipid chromatograms were sharper, possibly indicating the presence of a less complex mixture of triglycerides. In fact, the elution characteristics of this peak were remarkably similar to those observed for the HMG run under the same conditions (unpublished). Similarities in the fatty acid composition of the HMG (9) and the membrane triglyceride fraction obtained here further suggest that the HMG is the principal triglyceride fraction of the fat-globule membrane.

CONCLUSIONS

The following classes of lipids have been identified as constituents of the lipid fraction of the fat-globule membrane of normal bovine milk: carotenoids, cholesterol esters, cholesterol, and possibly one of its intermediates—squalene, triglycerides, mono-diglycerides, phospholipids and, in some instances, unesterified fatty acids.

Carotenoids and the squalene-like compound were not recovered from the ether-washed membrane preparation (Scheme II).

The differences encountered in the quantitative and qualitative composition between membrane preparations, principally the concentration of protein, phospholipids, and unesterified fatty acids, were attributed to the specific treatments employed in the isolation of the membrane lipids.

Similarities in the chromatographic elution characteristics and the fatty acid composition between the membrane triglyceride fraction and the high-melting glyceride fraction (HMG) suggest that the membrane triglyceride is composed principally of the HMG.

REFERENCES

(1) Heinemann, B. The Relation of Phospholipids to Fat in Dairy Products. J. Dairy Sci., 22: 707. 1939.

(2) Hirsch, J., and Ahrens, E. H. The Separation of Complex Lipid Mixtures by the Use of Silicic Acid Chromatography. J. Biol. Chem., 233: 311. 1958.

(3) Jenness, R., and Palmer, L. S. Substances Adsorbed on the Fat Globules in Cream and Their Relation to Churning. VI. Relation of the High-Melting Triglyceride Fraction to Butterfat and the "Membrane." J. Dairy Sci., 28: 653. 1945.

(4) Jensen, R. G., and Morgan, M. E. Estimation of the Monoglyceride Content of Milk. J. Dairy Sci., 42: 232. 1959.

(5) Mulder, H., Menger, J. W., and Koops, J. The Surface Layers of Milk Fat Globules. II. The Phospholipid Content of the Surface Layers. Netherlands Milk and Dairy J., 11: 263. 1957.

(6) Patton, S., and Keeney, P. The High-Melting Glyceride Fraction from Milk Fat. J. Dairy Sci., 41: 1288. 1958.

(7) RIMPILA, C. E., AND PALMER, L. S. Substances Absorbed on the Fat Globules in Cream and Their Relation to Churning. IV. Factors Influencing the Composition of the Adsorption "Membrane." J. Dairy Sci., 18: 827. 1935.

(8) SMITH, L. M., AND JACK, E. L. The Unsaturated Fatty Acids of Milk Fat. II. Conjugated and Nonconjugated Constituents. J. Dairy Sci., 37: 390. 1954.

(9) THOMPSON, M. P., BRUNNER, J. R., AND STINE, C. M. Characteristics of High-Melting Triglyceride Fractions from the Fat-Globule Membrane and Butteroil of Bovine Milk. J. Dairy Sci., 42: 1651. 1959.

(10) WHITE, R. F., EATON, H. D., AND PATTON, S. Relationship Between Fat Globule Surface and Carotenoid and Vitamin A Content in Milk in Successive Portions of a Milking. J. Dairy Sci., 37: 1. 1954.

MIGRATION OF PHOSPHATIDES IN PROCESSING DAIRY PRODUCTS

GEORGE R. GREENBANK AND MICHAEL J. PALLANSCH

Dairy Products Laboratory, Eastern Utilization Research and Development Division, USDA
Washington, D. C.

SUMMARY

The effect of stirring, pasteurization, homogenization, and condensing on the migration of the phosphatides from the fat globule membrane to the skimmilk has been studied. Any form of agitation apparently causes a migration. The greatest change in the distribution of the phosphatides occurs during condensing, where the turbulence encountered is great and prolonged. Homogenization at or below 2,000 p.s.i. causes a migration of phosphatides away from the fat globule surface, but at higher pressures the migration is reversed until at a pressure of 8,000 p.s.i. the amount of phosphatide associated with the fat phase is approximately equal to that in the untreated milk.

The material located on or near the surface of the fat globule in milk has been under investigation for over a century (1). A recent review (7) of the major work carried out during this period indicates that an adequate physical picture of the milk constituents in this interfacial zone and the changes they undergo during processing is still lacking.

The limitation of knowledge in this area necessitates added information to comprehensively and concisely explain the effect of the changes in the fat globule membrane during processing on the properties of finished dairy products.

This paper presents quantitative information on the changes in the concentration of the phosphatides associated with the fat globules caused by the various operations used in the processing of dairy products. Particular emphasis is centered on those operations utilized prior to desiccation in the production of foam-dried whole milk, since the chemical and physical basis for the unique properties of this material have been under extensive investigation in these laboratories for a considerable time.

MATERIALS AND METHODS

Total phosphatide concentration associated with the intact fat globules isolated by centrifugation was determined prior to each processing step. After processing, the phosphatide concentration in the centrifugally isolated fat phase was redetermined and compared with values obtained using untreated milk. All reported values of phosphatide concentration in the skimmilk fractions were obtained by difference.

All work was carried out using fresh morning milk from a herd at the Agricultural Research Center, Beltsville, Maryland. The milk was delivered to the laboratory in full, slosh-proof cans and standardized to 3.3% fat before experimental use.

Received for publication March 21, 1961.

Centrifugation was used to isolate the fat phase from samples of fresh and processed milk. Concentrated or dried products were reconstituted to original solids concentration with distilled water before centrifugation. Unhomogenized samples were centrifuged in a field of 515 × G for 2 hr. at 4.4° C. All homogenized samples were centrifuged in a field 20,000 G for 1 hr. at 4.4° C. In these instances both the cream layers and the sediment were collected for phosphatide determinations.

Phosphatide was determined by assaying for phosphorus in the ash obtained from a sample of the lipids extracted by the standard Mojonnier procedure. Three extractions were used instead of the prescribed two. Approximately 3 g. of the extracted and dried lipid was used for each analysis. The accurately weighed samples mixed with 3 g. ashless filter papers were ashed in Vycor evaporating dishes at 550° C. The resultant ash was dissolved in 5 ml. of 1:1 HCl with H_2O v/v and quantitatively transferred to 100-ml. volumetric flasks. After bringing up to volume with distilled water, the phosphorus in 5-ml. aliquots was determined by the Kuttner and Cohen method (8). Optical densities of the colored solutions were determined, using a Klett-Summerson colorimeter equipped with a 5,000A filter. The phosphorus content of the extracted lipids in milligrams per gram fat was multiplied by 25.0 to give milligrams of phosphatide per gram fat (8).

In pilot plant operations use was made of a Cherry-Burrell Model CC35 spray pasteurizer, a Mallory type heater, and a Manton-Gaulin Model K3-75 homogenizer. Where required, milk was concentrated using a Rogers single-effect pan.

RESULTS

The effect of the temperature of the milk on the migration of phosphatides from the fat globule surface induced by simple stirring is shown in Table 1. These data were obtained by stirring samples of raw whole milk for 1 min. in a modified Waring blendor (3), at temperatures of 10, 20, 40, and 50° C. At 10° C. very little phosphatide moved from the fat globule surface during the stirring period. At 20° C. nearly 46% of the phosphatide initially associated with the fat phase had migrated into the aqueous phase. As temperatures were raised above this point, a slight but consistent decrease in migration was noted.

TABLE 1

Migration of phosphatides in milk during stirring at various temperatures

Treatment	Phosphatide concentration % total in cream	Phosphatide concentration % total in skim	% Total phosphatides migrating from cream during stirring period
No agitation 10° C.	64.4	35.6	0.0
1 min. stirring 10° C.	59.2	40.8	5.2
1 min. stirring 20° C.	34.9	65.1	29.5
1 min. stirring 40° C.	40.3	59.7	24.1
1 min. stirring 50° C.	40.9	59.1	23.5

TABLE 2

Migration of phosphatides in milk during pasteurization

Pasteurization	Mg. phosphatide/100 g. 3.3% fat milk					% Total phosphatides migrating from cream during pasteurization
		Cream		Skim		
	Whole milk	Raw	Pasteurized	Raw	Pasteurized	
145° F.—30 min.	20.4	14.1	11.2	6.3	9.2	14.2
145° F.—30 min.	21.0	13.6	11.2	7.4	9.8	11.4
145° F.—30 min.	19.8	13.2	9.9	6.6	9.9	16.7
145° F.—30 min.	19.7	13.6	10.2	6.1	9.5	17.3
165° F.—15 sec.	21.1	13.9	12.6	7.2	8.5	6.2
165° F.—15 sec.	22.8	14.2	13.2	8.6	9.6	4.4
165° F.—15 sec.	20.5	14.2	12.9	6.3	7.6	6.3
165° F.—15 sec.	19.8	13.9	12.9	5.9	6.9	5.1

Milk samples pasteurized by various time and temperature combinations may be bacteriologically similar but physically different. Table 2 presents data showing the extent of phosphatide migration from the fat globule surface during two different pasteurizing treatments. Even though the agitation in the spray pasteurizer was relatively mild, its extended duration effected more change than the short period of high turbulence encountered in the Mallory type heater.

Condensation in a single-effect evaporator exposes milk to violent agitation for considerable periods of time. This type of processing also causes a shift of phosphatide from the fat globules to the aqueous phase, as shown in Table 3. The longer evaporating times used to produce the 50% solids concentrates effected the greatest change. The variance in results obtained with different samples of milk can be correlated with the time required by the operator to obtain the stated solids concentration.

A study of the effect of homogenization on the distribution of phosphatides in milk was complicated because the treatment produces fat protein complexes which sediment in a centrifugal field (2). As the homogenization pressure increased, the amount of fat recovered as cream decreased (4). Table 4 shows the

TABLE 3

Migration of phosphatides during the condensation of milk pasteurized 165° F.—15 sec.

Treatment	Phosphatide concentration % total				% Total phosphatides migrating from cream during condensation
	Pasteurized milk		Concentrate		
	Cream	Skim	Cream	Skim	
Pasteurization plus	56.2	43.8	31.0	69.0	25.2
condensation to	55.6	44.4	46.0	64.0	19.6
26% total solids	52.3	47.7	27.0	63.0	15.7
Pasteurization plus	49.1	50.9	21.1	78.9	28.0
condensation to	53.9	46.1	17.7	82.3	36.2
50% total solids	53.5	46.5	19.0	81.0	34.5

TABLE 4

Change in distribution of phosphatides during the homogenization of milk before and after concentration

Processing	Phosphatide concentration (% Total phosphatides in milk)			Change in % total phosphatide in skim fraction during processing
	Cream	Sedimenting fat protein complex	Skim	
Untreated milk	65.1	0.0	34.9	0.0
Homogenized—2,000 p.s.i.	34.3	14.4	51.3	+16.4
Homogenized—4,000 p.s.i.	31.4	18.3	50.3	+15.4
Homogenized—6,000 p.s.i.	33.1	20.6	46.3	+11.4
Homogenized—8,000 p.s.i.	34.9	25.7	39.4	+ 4.5
50% solids concentrate homogenized—2,000 p.s.i.	30.9	22.9	46.2	+11.3
50% solids concentrate homogenized—4,000 p.s.i.	24.6	32.0	43.4	+ 8.5
50% solids concentrate homogenized—6,000 p.s.i.	26.3	61.7	12.0	−22.9
50% solids concentrate homogenized—8,000 p.s.i.	24.0	63.4	12.6	−22.3

effect of homogenization on the distribution of the phosphatides in milk. Low-pressure homogenization increases the phosphatide concentration in the aqueous phase. Higher pressures reduce the migration to the aqueous phase and increase the amount of phosphatide in the sedimenting fat protein complex. Homogenizing concentrated milk intensifies these effects, as shown. It is of interest that in single-strength milk homogenized at 8,000 lb. p.s.i. the phosphatide concentration in the skim is nearly the same as in untreated milk (Figure 1).

DISCUSSION

The migration of phosphatides from the fat globule surface zone to the aqueous phase apparently occurs to some extent whenever milk undergoes mechanical agitation. This effect, possibly first noted by Jenness (6), later studied in more detail and discussed by Greenbank and Pallansch (3), has recently been further verified by the work of Koops and Tarassuk (9).

These observations all serve to emphasize the fact that the design of dairy equipment can influence the physical structure of the products obtained therefrom. Those pieces of apparatus which subject the milk moving through them to the highest level of mechanical agitation should deplete the phosphatides associated with the fat globules to the greatest extent.

Whether this translocation of the phosphatides can serve as a basis for dairy product improvement is still open to question. However, Thurston and coworkers (12) definitely established the fact that homogenization at moderate pressures and stirring prevent the development of oxidized flavors in market

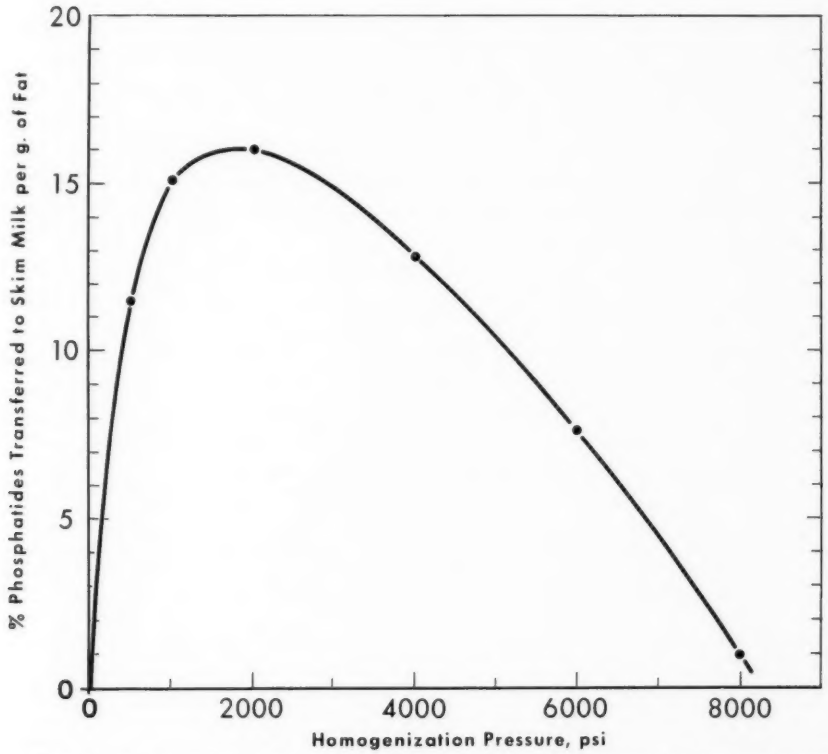


FIG. 1. The migration of the phosphatides of whole milk, with increasing homogenization pressures.

milk. Holm, Greenbank, and Deysher (5) found that milk powder made from homogenized milk had a longer storage life than similar products made from unhomogenized material. These latter authors assumed that the improved keeping quality resulted from a decrease in uncovered fat in the powder. In both instances cited, the observed increase in oxidative stability may have resulted from the redistribution of the phosphatides by the homogenization treatment employed (Figure 1).

It is possibly significant that some groups are presently improving the dispersibility of spray-dried whole milk by the addition of phosphatides to the product. It is possible that violent agitation of the milk during the processing steps prior to drying would achieve similar results.

Along with its practical aspects, the data presented in this paper have some theoretical significance. The relative ease with which phosphatides can be removed from the fat globule surface demonstrates that a large fraction of the phosphatides initially associated with the fat in fresh milk are held by weak forces.

The ordered picture of the fat globule surface, presented by some authors (7), in which a phosphatide film on the fat surface is covered by layers of protein to produce a fat globule membrane, can be questioned.

It is more reasonable to suspect that the bulk of the fat globule surface may be covered by a film of a highly surface-active protein whose exposed groups interact with phosphatides, and phosphatide containing protein-lipid complexes such as described by Morton (11). The force of mechanical agitation plus the thermal agitation present at moderate temperatures is sufficient to rupture a large percentage of these bonds.

When the surface of the fat phase is greatly extended by high-pressure homogenization a situation is created in which new lipid protein complexes are formed which again bind additional phosphatides.

The effect of phosphatide distribution on the properties of foam-dried and spray-dried whole milk, and the factors responsible for the formation of sedimentable phosphatide containing lipid-protein complexes during high-pressure homogenization, are being investigated in the Dairy Products Laboratory of the Eastern Utilization Research and Development Division.

REFERENCES

(1) Ascherson, F. M. On the Physiological Utility of the Fats and a New Theory of Cell Formation Based on Their Cooperation and Suggested by Several New Facts. Arch. Anat. u. Physiol., Leipzig, 7: 44. 1840.

(2) Fox, K. K., Holsinger, Virginia H., Caha, Jeanne, and Pallansch, M. J. Formation of a Fat Protein Complex in Milk by Homogenization. J. Dairy Sci., 43: 1396. 1960.

(3) Greenbank, G. R., and Pallansch, M. J. Migration of the Phosphatides in the Processing of Dairy Products. J. Dairy Sci., 42: 904. 1959.

(4) Greenbank, George R., Wright, Philip A., Deysher, Edgar F., and Holm, George E. The Keeping Quality of Samples of Commercially Dried Milk Packed in Air and in Inert Gas. J. Dairy Sci., 29: 55. 1946.

(5) Holm, G. E., Greenbank, George R., and Deysher, E. F. The Effect of Homogenization, Condensation and Variations in Fat Content of a Milk on the Keeping Quality of Its Powder. J. Dairy Sci., 8: 515. 1925.

(6) Jenness, Robert, and Palmer, L. S. Substances Adsorbed on the Fat Globules in Cream and Their Relation to Churning. V. Composition of the "Membrane" and Distribution of the Adsorbed Substances in Churning. J. Dairy Sci., 28: 611. 1945.

(7) King, N. The Milk Fat Globule Membrane. Tech. commun. No. 2, Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England. 1955.

(8) Koops, J. Isolation of Phosphatides from Butter Serum. Netherlands Milk Dairy J., 11: 43. 1957.

(9) Koops, J., and Tarassuk, N. P. The Effect of Various Processing Treatments on the Partition of Phosphatides Between the Fat Phase and the Milk Plasma. Netherlands Milk Dairy J., 13: 180. 1959.

(10) Kuttner, Theodore, and Cohen, Harriet R. Micro Colorimetric Studies. I. A Molybdic Acid, Stannous Chloride Reagent. The Micro Estimation of Phosphate and Calcium in Pus, Plasma, and Spinal Fluid. J. Biol. Chem., 75: 517. 1927.

(11) Morton, R. K. The Lipoprotein Particles in Cow's Milk. Biochem. J., 57: 231. 1954.

(12) Thurston, L. M., Brown, W. Carson, and Dustman, R. B. Oxidized Flavor in Milk. II. The Effects of Homogenization, Agitation and Freezing of Milk on Its Subsequent Susceptibility to Oxidized Flavor Development. J. Dairy Sci., 19: 671. 1936.

EFFECTS OF DIRECT STEAM HEATING AND VACUUM TREATMENTS ON THE CHEMICAL COMPOSITION OF MILK WITH ESPECIAL REFERENCE TO SUBSTANCES INVOLVED IN OXIDIZED FLAVOR DEVELOPMENT [1]

D. H. KLEYN [2] AND W. F. SHIPE, JR.
Department of Dairy and Food Science, Cornell University, Ithaca, New York

SUMMARY

Milk was heated with live steam to from 190 to 240° F. and subjected to vacuum treatments of approximately 9 in. of mercury in one vacuum chamber and 20 in. of mercury in a second vacuum chamber. The direct steam heating and vacuum equipment were used in conjunction with a H.T.S.T. pasteurizing system and a homogenizer.

The combined steam heating and vacuum treatment was found to produce a significant denaturation of whey proteins, as measured by the increase in the activity of sulfhydryl groups and the decrease in whey protein nitrogen. The decrease in whey protein nitrogen was shown to be approximately that which would be expected from published data on the effect of indirect heating. However, the extent of activation of sulfhydryl groups was lower than had been expected. The possibility that the vacuum treatment removed some of the volatile sulfhydryls was discussed. Increased activation of sulfhydryl groups was observed upon homogenization, particularly after direct steam heating. It was suggested that sulfhydryl activation may be a factor contributing to the high resistance of homogenized milk to oxidized flavor development.

The lack of correlation between the nitroprusside test and cooked flavor is believed to indicate that the nitroprusside test is less sensitive than flavor perception, or that cooked flavor is not necessarily due to nitroprusside reactive substances.

A high retention of ascorbic acid was observed in milk subjected to direct steam heating and vacuum treatment. This effect, along with resistance to oxidized flavor development, was noted even in milks to which copper was added. The removal of oxygen by the vacuum treatment and the activation of sulfhydryl groups by the heat treatment were considered to be contributing factors. Homogenization was also shown to contribute to the stability of ascorbic acid and flavor. However, homogenization alone was less effective than the combination of homogenization with steam heating–vacuum treatment.

The removal of off-flavors is a well-known effect of the combination of direct steam heating and vacuum treatment of milk. However, the effect of such treatment on the other properties of milk has not been given much consideration.

On the basis of studies using conventional heating, i.e., indirect heating, one could predict that certain components of the milk would be affected by direct steam heating. For example, heating to 90° C. (194° F.) for 30 min. has been shown (3) to bring about an increase in the activity of sulfhydryl groups; and heating to 95° C. (203° F.) for 10 min. was found (11) to produce over 90% denaturation of the whey proteins. On the other hand, the vacuum treatment might remove some of the sulfhydryl groups produced by the heat treatment. Consequently, one objective of this study was to determine if the combi-

Received for publication February 3, 1961.

[1] These data were taken in part from a thesis presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Cornell University.

[2] Present address: General Foods Corporation, 555 S. Broadway, Tarrytown, New York.

nation direct steam heating and vacuum treatment had any unique effect on the whey proteins.

On theoretical grounds, one would expect the vacuum treatment to lower the oxygen content. Furthermore, on the basis of previous findings (5, 12, 13, 17, 28), it would seem reasonable to assume that a lowered oxygen level would retard the destruction of ascorbic acid and the development of oxidized flavor. From the findings of Gjessing and Trout (9) one would predict that high-heat treatment would retard oxidation. Therefore, a second objective of this study was to determine the extent and nature of the combined effect of the steam heating and vacuum treatment on oxidative reactions in milk.

PROCESSING EQUIPMENT AND PROCEDURE

Commercially available processing equipment was used in this study. A conventional high-temperature–short-time (H.T.S.T.) pasteurizer unit and homogenizer were used. The vacuum treatment was accomplished with a double-chamber Cherry-Burrell [3] Aro-Vac Model AV-A unit. In an initial experiment, steam was infused into the bottom of the first vacuum chamber. In this experiment the milk was heated to 190 ± 2° F. in the first chamber. In subsequent experiments, an Ultra-High-Temperature (U.H.T.) direct steam injector located on the input side of the first vacuum chamber was used to heat the milk to from 200 to 240° F. The combined steam heating and vacuum treating unit is subsequently referred to as the steam-vacuum unit. Milk processed in this unit is subsequently referred to as treated milk.

Milk to be steam vacuum–treated was first pasteurized at approximately 165° F. in the H.T.S.T. unit. Steam at about 15 p.s.i. pressure was introduced directly into the milk, bringing it to the desired temperature. The temperature in the first vacuum chamber was maintained at 190° F., irrespective of whether the steam was injected in the U.H.T. heater or infused into the chamber. After passing through the first chamber, the milk was drawn into the second chamber by a higher vacuum (9 in. vs. 20 in.), where it was cooled to about 160° F. A centrifugal pump was used to pump the milk to the homogenizer, where it was homogenized at 1,500 p.s.i. pressure. The homogenizer, serving also as a booster pump, pumped the milk to the H.T.S.T. unit. The milk was then cooled in the regenerative and cooling sections of the pasteurizer to a temperature of about 40° F. The rated capacity of the steam-vacuum unit was 8,000 lb/hr, but because the homogenizer and H.T.S.T. unit had lower capacity, the equipment was operated at about 4,000 lb/hr. The approximate average holding times in the various sections of the processing system were calculated from the average velocity of the milk. These holding times are presented in Table 1, since they are considered to be pertinent to the interpretation of the data.

For comparative purposes, some milk samples were obtained which had merely been pasteurized at 163 ± 1° F. for 16 sec. Other samples were pasteurized and homogenized without passing through the steam-vacuum unit.

[3] Cherry-Burrell Corporation, Cedar Rapids, Iowa.

TABLE 1

Time-temperature treatments of milk [a]

Section of system	Length of tube	Cross section of tube area	Calculated average holding time	Temperature
	(ft.)	*(sq. ft.)*	*(sec.)*	*(° F.)*
H.T.S.T. holding tube	34	0.01072	20	163–165
Flow div. valve to first sampling valve [b]	20	0.01072	12	165
First sampling valve to U.H.T. Heater	8	0.01072	5	165
U.H.T. Heater to first vacuum chamber	3.6	0.00444	0.9 [c]	200–240
Second vacuum chamber to second sampling valve [b]	9	0.01072	5+	160
Second sampling valve to homogenizer	8	0.01072	5	160
Homogenizer to H.T.S.T. unit	15	0.01072	9	160+
Flow div. valve to H.T.S.T. unit via homogenizer	37	0.01072	22	163

[a] Velocity values were calculated by using the formula $Q = A \times V$ where Q = rate of flow (4,000 lb. per hour = 0.0178 cu. ft/sec), A = area of tube (as shown in table), and V = velocity (ft/sec). The average holding time was then determined by dividing the velocity into the length of tube.

[b] The first sampling valve was used to collect the pretreated samples and the second sampling valve to collect the treated samples.

[c] The time it took milk to pass from a point 13 in. before the U.H.T. heater to the bottom of the second chamber of the steam-vacuum unit was determined by Peterson (25). He found that the average holding time in this section was approximately 4 sec.

Sampling procedures. Fresh (24-48 hr.), cooled (40° F.), raw milk samples were collected in clean, sanitized flasks from a 1,500-gal. storage tank. In each trial the processed samples were obtained from this same supply of milk. Regular milk (i.e., milk that had merely been pasteurized) was collected as the bottled product. Homogenized milk refers to pasteurized and homogenized milk. This milk was collected in clean sanitized flasks from a valve located immediately beyond the cooling section of the H.T.S.T. unit or from the surge tank.

Sampling valves located immediately before and after the steam-vacuum unit were used to obtain samples subsequently referred to as pretreated and treated, respectively. For the whey protein studies these two samples were allowed to flow from the sampling valve through a coil of aluminum tubing immersed in an ice bath. The temperature of the milk as it flowed into a clean, sterile flask was approximately 80° F. It was further cooled by immediately immersing the flask in an ice water bath. For the oxidation studies, these two samples were collected in sterile flasks and immediately stoppered and cooled in ice water. Milk which had been subjected to the complete H.T.S.T.—steam-vacuum-homogenizer treatment is referred to as the homogenized-treated milk. It was collected as the bottled product, except for the study involving oxidized flavor development. In this case, it was taken from a valve located immediately beyond the cooling section of the H.T.S.T. unit.

All samples not analyzed fresh were transferred to clean sanitized half-pint bottles, tightly stoppered, and stored in the dark at 40° F. In the studies involving a comparison of cooked flavor and sulfhydryl activation, analyses were

made within 1 hr. after processing. All other sulfhydryl measurements on fresh samples were made within 3 hr. In the studies of whey protein denaturation analyses were made within 24 hr.

Procedure for oxygenation studies. A sample of homogenized-treated milk was collected in a sterile flask and divided into two aliquots immediately after processing. One aliquot, which served as a control, was poured directly into clean, sterile half-pint bottles. The other aliquot was saturated with sterile oxygen by diffusing oxygen through a fritted glass disc placed in the bottom of the flask for a period of 5 min. The oxygen had been sterilized by passing it through a 1-ft. length of pipe packed with sterile glass wool. This milk was then poured into clean, sterile half-pint bottles and stored along with the other samples.

Procedure for studies involving added copper. In the first trial, samples were divided into three lots immediately after collection. One lot was transferred to clean, sterile half-pint bottles. The other two lots were transferred to clean, sterile bottles containing approximately 0.5 and 1.2 ml. of a 0.04% copper sulfate solution. These amounts of copper were used to yield 0.2 and 0.5 p.p.m. of copper in the respective milks. The same procedure was followed in the second trial, except that higher levels (i.e., 2.5 and 5.0 p.p.m.) of copper were used. A 0.4% solution of copper sulfate was used, so that only 0.6 and 1.2 ml. were required.

The samples were judged at periodic intervals by experienced judges. Three judges participated in the first trial and four judges in the second trial. Ascorbic acid analyses also were performed. The samples were removed from the refrigerator approximately 30 min. before judging. An aliquot was taken and tested immediately for ascorbic acid. The samples were coded by a technician who was not a member of the taste panel. The judges were instructed to criticize the milks for oxidized flavor only, using the following scoring system: 0, no oxidized flavor detected; ?, oxidized flavor doubtful; 1, slight oxidized flavor; 2, definite oxidized flavor; 3, strong oxidized flavor; 4, very strong oxidized flavor.

The fresh samples were judged within 2 hr. after the copper had been added.

METHODS OF ANALYSIS

Argentometric-amperometric titration of sulfhydryl groups. The method and apparatus employed for the titration of sulfhydryl groups were essentially those of Kolthoff and Harris (18), as adapted to skimmilk by Hutton and Patton (15). The raw milk was titrated in an alcoholic medium, whereas the heated milks were titrated in an aqueous medium. According to Hutton and Patton, the total number of sulfhydryl groups capable of activation by heat can be determined in raw skimmilk by titrating in an alcoholic medium. They reported that titrations in aqueous media measured the same quantity of sulfhydryl groups in heated milk as nitroprusside and thiamine disulfide. The latter two reagents are considered to measure only the sulfhydryl groups activated by heat treatment.

The galvanometer of a Sargent Manual Polarograph, Model III, was employed in the titration. It was adjusted so that each millimeter of the galvanometer scale was equal to 0.063 microamperes. The rotating platinum electrode was rotated at approximately 130 r.p.m. The mercury–mercuric iodide reference electrode was prepared in a 125-ml. Erlenmeyer flask as described by Kolthoff and Harris (18).

The method of Parks and Lykken (23) was used to standardize the 0.001 *M* silver nitrate employed in the titration. The electrode was standardized before each series of titrations against either a freshly prepared 0.001 *N* solution of glutathione or *n*-dodecyl mercaptan. Quantitative measurement of either of these compounds indicated that the apparatus was operating satisfactorily.

Information on the reliability and significance of the argentometric-amperometric titrations has been published by a number of workers (1, 2, 4, 19, 32). Although there is some question as to the exact interpretation of data obtained with this method, the authors felt that it would provide useful information on sulfhydryl activation.

Nitroprusside test. The procedure of Josephson and Doan (17), as modified by Patton and Josephson (24), was used for this test.

Nitrogen analyses. The wheys for the nitrogen analyses were prepared by the method of Rowland (26). The digestions and distillations were performed by a slight modification of a procedure developed by Herrington (14) for the total protein nitrogen content of milk. The titrations were performed by an achromatic technique, recommended for small quantities of ammonia (20). The accuracy of the method was checked by running an analysis upon a 104.5-mg. sample of C.P. DL-lysine. There was a recovery of 105.0 mg. of lysine, indicating the accuracy of the method.

For these investigations, the samples were analyzed for their noncasein nitrogen and nonprotein nitrogen contents. The whey protein nitrogen was calculated by subtracting the nonprotein nitrogen from the noncasein nitrogen. The percentage of whey protein coagulated was calculated by assuming that the whey protein in the raw milk was entirely in its natural form. The analyses were performed within 24 hr. after processing.

Reduced ascorbic acid. Titrations with 2,6-dichlorophenolindophenol of metaphosphoric-trichloroacetic acid filtrates, prepared according to Josephson and Doan (17), were used to determine reduced ascorbic acid. The dye was standardized against ferrous ammonium sulfate, as recommended by Stewart and Sharp (33).

Oxygen. Dissolved oxygen was determined quantitatively by the ascorbic acid oxidase method of Sharp *et al.* (28). The dye was again standardized, as recommended by Stewart and Sharp (33). The L-ascorbic acid was procured from Eastman Organic Chemicals. The ascorbic acid oxidase was prepared from cucumbers, pervaporation being employed to concentrate the juice.

Vitamin C. Total vitamin C was measured by the method of Gunsalus and Hand (10).

RESULTS AND DISCUSSION

Effect of treatment on the whey proteins. The initial phase of the whey protein study was designed to determine the effect of processing on sulfhydryl activation.

In the first experiment, the extent of activation occurring in regular, homogenized, and homogenized-treated milk was determined. The sulfhydryl groups capable of being activated by heat were measured by titrating the raw milks in 95% ethyl alcohol. The results obtained for two different samples of milk were 0.131 and 0.156 m.eq/liter, expressed as cysteine hydrochloride. There was a very low level of activation occurring in the corresponding regular milks, i.e., about 0.002 and 0.004 m.eq/liter. The homogenized milk was found to have a slightly higher degree of activation, i.e., 0.006 and 0.007 m.eq/liter for the respective milks, but the total degree of activation was still very low. The added holding time of about 22 sec. could be responsible for the observed higher activation in the homogenized milk, but homogenization may also be a contributing factor. The homogenized-treated milk had a much higher degree of sulfhydryl activation, i.e., 0.027 m.eq/liter for both samples. Undoubtedly the heat treatment (i.e., 200 $\pm$ 1° F.) received by the milks in the U.H.T. heater contributed towards the higher values in the homogenized-treated milks but, as is noted below, homogenization also appears to be a contributing factor.

The second experiment was performed to determine the extent of sulfhydryl activation occurring in milk as it passed through the processing system; and to observe the rate of depletion of the activated sulfhydryl groups in the various milks on storage. In the hopes of obtaining a greater degree of sulfhydryl activation than in the previous experiment, the treated samples were subjected to 230° F. in the U.H.T. heater.

The results in Table 2 show that there was a negligible amount of activation occurring in the pretreated milk, with the exception of the milk in Trial 1. Only the fresh samples of pretreated milk were tested for sulfhydryl groups, since the degree of activation was low. The initial sulfhydryl values for the treated milk range from 0.021 to 0.031 m.eq/liter expressed as cysteine hydrochloride. These values indicate that the heat treatment received by the milk in the U.H.T. heater is activating the sulfhydryl compounds. However, these values are much lower than the 19-22 mg/liter range (i.e., .121-.139 m.eq/liter) reported by Boyd and Gould (3) for milk samples at the time they reached 90° C. (194° F.). The reason for the lower values in the case of treated milk probably is because the milk is held at the high temperature for only a few seconds in the steam-vacuum unit, whereas it took approximately 8 min. for Boyd and Gould to heat milk to 90° C. and cool it to 75° C. (167° F.).

The initial sulfhydryl values for the homogenized-treated milk samples were 0.017, 0.007, and 0.013 m.eq/liter higher than the sulfhydryl values for the corresponding treated milk samples. These differences were much greater than the difference between the sulfhydryl values of the regular and homogenized milks, as reported in the first experiment. A possible explanation for this increased effect of homogenization is that the high heat treatment received by the

TABLE 2

Effect of processing on the activation of sulfhydryl groups and their depletion during storage

Trial	Time	Titrating conditions [a]	Sulfhydryl values (Expressed as m.eq. of cysteine-HCl/liter) Raw	Pre-treated	Treated [b]	Homogenized-treated [b]
1	Fresh	EtOH	0.166	.....	.....	.....
	Fresh	H_2O	.....	0.012	0.031	0.048
	48 hr.	H_2O	.....	.....	.025	.029
	96 hr.	H_2O	.....	.....	.017	.021
	144 hr.	H_2O	.....	.....	.005	.017
	2 wk.	H_2O	.....	.....	.000	.000
2	Fresh	EtOH	0.154	.....	.....	.....
	Fresh	H_2O	.....	0.004	0.023	0.030
	48 hr.	H_2O	.....	.....	.015	.017
	96 hr.	H_2O	.....	.....	.004	.022
	144 hr.	H_2O	.....	.....	.000	.008
	2 wk.	H_2O	.....	.....	.000	.008
3	Fresh	EtOH	0.192	.....	.....	.....
	Fresh	H_2O	.....	0.006	0.021	0.034
	48 hr.	H_2O	.....	.....	.018	.019
	96 hr.	H_2O	.....	.....	.008	.014
	144 hr.	H_2O	.....	.....	.003	.012
	2 wk.	H_2O	.....	.....	.000	.004

[a] The raw milks were titrated in 95% ethanol and the values indicate the total number of sulfhydryl groups capable of activation by heat treatment.

[b] Treated samples were heated to 230 ± 2° F. in the U.H.T. heater.

milk in the U.H.T. heater affects the whey proteins so that the sulfhydryl groups are extremely easily activated by homogenization. Of course, the added period of time (about 14 sec.) that the homogenized-treated milk was held at 160° F. may be partially responsible for the activation.

The published evidence concerning the effect of homogenization on proteins is not clear cut. Menefee *et al.* (21) reported that homogenization of milk at normal and abnormal pressures produced no significant changes in the nitrogen distribution. Shahani and Sommer (27), on the other hand, observed that the globulin nitrogen decreased by about 20% in milk pasteurized at 155° F. for 30 min. and an additional 10% upon homogenization at 2,500 p.s.i. pressure. They believed that the increased loss caused by homogenization probably occurs by virtue of the heat of friction in the process, although the violent agitation and impacts in the homogenizer valve were also considered as possible factors. Incidentally, the sulfhydryl groups activated in milk by heat treatment are derived from the globulin fraction, according to Hutton and Patton (15).

If homogenization does, in fact, bring about some sulfhydryl activation, this may explain the resistance of homogenized milk to oxidized flavor. Forster and Sommer (8), in 1951, theorized that relatively few sulfhydryl groups may be necessary to provide for sufficient protein oxidation to protect milk against oxidized flavor development. Tarassuk and Koops (34) have recently proposed

an alternate explanation for homogenization retarding oxidized flavor development. They claimed this effect was due to the large increase in fat globule surface and the proportional decrease in concentration of phospholipids and copper per unit of newly formed fat globule surface area.

The sulfhydryl groups in the treated milks had almost completely disappeared in six days. On the other hand, a measurable quantity of sulfhydryl groups was observed in the homogenized-treated milks after 2 wk. in two of the three trials. The observation that the sulfhydryl groups disappear on storage is compatible with the thoery that they are serving as antioxidants.

A third experiment was performed primarily to find out the extent of denaturation occurring in milk processed at different temperatures in the steam-vacuum unit. A secondary reason was to observe whether homogenization increases the extent of denaturation. In this experiment, the denaturation was measured in terms of the decrease in whey protein nitrogen.

The results of this experiment are shown in Table 3. A comparison of the denatured whey protein percentages of the individual pretreated samples con-

TABLE 3

Effect of processing on whey protein denaturation

Sample	Temperature of treatment	Whey protein nitrogen	Whey protein denatured (%)	
			Compared to raw milk	Compared to pretreated milk [a]
	(° F.)	(mg/liter)		
Trial No. 1				
Raw		0.942	.....	.....
Pretreated		0.670	27.9	.....
Treated	200	0.634	32.6	4.7
Treated	210	0.632	32.9	5.0
Treated	220	0.598	36.5	8.6
Treated	230	0.603	36.0	8.1
Trial No. 2				
Raw		0.808	.....	.....
Pretreated		0.722	10.6	.....
Treated	200	0.639	20.9	10.3
Treated	210	0.620	23.3	12.7
Treated	220	0.580	28.2	17.6
Treated	230	0.573	29.1	18.5
Trial No. 3				
Raw		0.816	.....	.....
Pretreated		0.648	20.6	.....
Treated	200	0.577	29.3	8.7
Homogenized-treated	200	0.555	32.0	11.4
Treated	230	0.566	30.7	10.1
Homogenized-treated	230	0.548	32.8	12.2
Trial No. 4				
Raw		0.802	.....	.....
Pretreated		0.704	12.2	.....
Treated	230	0.578	27.9	15.7
Homogenized-treated	230	0.535	33.3	21.1

[a] These values represent the per cent whey protein denatured by and subsequent to the U.H.T. treatment.

firmed the observation reported by Jenness (16). He reported that the response to heat treatments varies among different lots of milk. However, as the milks were exposed to U.H.T. heat treatments, especially at 220° F. or above, they were found to lose some of their individuality, i.e., the percentage of whey proteins denatured falls into a much narrower range. For instance, the treated milks heated to 230° F. in the U.H.T. heater had a range of 27.9 to 36.0% of whey protein denatured. This range was relatively narrow in comparison to the range of 10.6 to 27.9% for the pretreated milks.

The extent of denaturation actually due to the U.H.T. heat treatment can be estimated by comparing the denaturation occurring in the pretreated samples with the denaturation in the treated samples. It is recognized that the treated milk has been exposed to 160° F. for a short period of time after leaving the U.H.T. heater. However, this added exposure probably does not appreciably affect denaturation. The denaturation produced by and subsequent to the U.H.T. treatment is shown in the last column of Table 3. These values indicate that the extent of denaturation increased with temperature. Moreover, the greatest change was observed between the temperatures of 210 and 220° F. This observation may have some bearing on the general observation that the long retention of cooked flavor begins in milk heated to 220° F. or higher in the U.H.T. heater.

Using the time-temperature relationships established by Harland and Ashworth (11) as a reference, it is possible to roughly estimate the extent of denaturation which should occur in U.H.T. heating. They observed that for every 13.5° F. increase in temperature, there was a tenfold decrease in the time required for a given amount of denaturation. It required 18 and 42 sec. at 175° F. for 5 and 10% denaturation to occur. If the tenfold decrease in time required for a given amount of denaturation is extended to 200° F., it will be seen that 0.9 and 2.1 sec. would be required for 5 and 10% denaturation to occur. Thus, the amount (7.9%) of denaturation occurring in the U.H.T. heater at 200° F. was roughly that which would be expected, since the calculated holding time in the U.H.T. heater was 0.9 sec.

The average amount of denaturation occurring in the U.H.T. heater for the four samples of treated milks heated to 230° F. was 13.1%. It required about 70 sec. for 15% denaturation to occur in milk heated at 175° F., according to Harland and Ashworth. The calculated amount of time for 10 and 15% denaturation to occur at 230° F. is 1.0 and 1.7 sec., respectively. Thus again, the amount (13.1%) of denaturation occurring in the U.H.T. heater at 230° F. is roughly that which would be expected.

The calculations above are only approximations, as several factors limit their accuracy. The holding time of 0.9 sec. is only a rough estimate. The rate of heating in the U.H.T. heater and cooling in the vacuum chambers is much more rapid than in the experiments of Harland and Ashworth. The extent of serum protein denaturation was determined by a Kjeldahl procedure, whereas Harland and Ashworth used a turbidimetric technique. Finally, their finding that a temperature increase of 13.5° F. yielded a tenfold increase in the rate of dena-

turation between 155 and 175° F. does not guarantee that the same relationship will hold at higher temperatures.

The results of Trials 3 and 4 show that the homogenized-treated milks have between 2.1 and 5.4% more denatured serum protein than the treated milks. As has been noted previously, the increased denaturation may be due to homogenization, or to the additional 14 sec. at 160° F. at which the homogenized-treated milk is held, or to both.

The fact that processing milk in the steam injection–vacuum system did result in a significant amount of protein denaturation may have practical value to cottage cheese manufacturers. Emmons *et al.* (7) have recently reported that a cottage cheese of good commercial quality can be manufactured from fluid skimmilk heated to 175° F. to denature the whey proteins. In addition, they have pointed out that there is an increased yield of cottage cheese made from skimmilk exposed to high heat treatments, presumably due to the incorporation of the denatured whey proteins into the curd. Another advantage of making cottage cheese from skimmilk exposed to direct steam heating and vacuum treatments would be the increased uniformity of flavor.

A comparison of cooked flavor with sulfhydryl activation as measured by the nitroprusside test and the argentometric-amperometric titration. Inasmuch as cooked flavor is believed to be associated with heat-activated sulfhydryl groups, the effects of direct steam heating and vacuum treatments on cooked flavor also were studied. The cooked flavor resulting from heat treatment of milk was first associated with heat-activated sulfhydryl groups by employment of the classical nitroprusside test (17). Therefore, this test was run in addition to the amperometric method.

The results of this experiment are shown in Table 4. However, they are not easily interpreted unless reference is first made to the literature. Josephson and Doan (17) reported a close correlation between the nitroprusside test and cooked flavor. In milks that were flash-heated to 180 and 190° F., distinctly cooked flavors were observed and the nitroprusside values were 2 and 5, respectively. Patton and Josephson (24) reported the nitroprusside values for skimmilks flash-heated to 174.1, 179.9, 190, and 199.9° F. to be 1, 2, 4, and 5, respectively. Townley and Gould (35), by using a photometric technique, which included bubbling nitrogen through milk during the heat treatment and for 30 min. thereafter, were able to quantitatively measure the volatile sulfides of milk. Momentary heating of milk at 95° C. (203° F.) liberated 0.158 mg/liter of volatile sulfides, yielded a nitroprusside test of four, and brought about a cooked flavor in the milk. They concluded that cooked flavor may be attributed to volatile sulfides, particularly hydrogen sulfide.

From the literature cited above, one would predict that the heating of milk to 210° F. or higher should yield a high nitroprusside value. However, as the results in Table 4 show, even heating to 240° F. yielded a low value, although the milk did have a very distinct cooked flavor. The sulfhydryl values ranged from zero m.eq/liter for pretreated milk to 0.031 m.eq/liter for treated milk heated to 240° F. As in previous experiments, the homogenized-treated milks

TABLE 4

Comparison of the intensity of cooked flavor with the degree of sulfhydryl activation

Sample	Temperature of treatment	Flavor [a]	Nitroprusside value [b]	Amperometric method	
				Titrating conditions [c]	Sulfhydryl value [d]
Trial No. 1	(° F.)				
Raw		Sl. feed	0	EtOH	0.173
Pretreated		Sl. feed	0	H_2O	0.000
Treated	210	Sl. cooked	1	H_2O	0.021
Treated	240	Strong cooked	1	H_2O	0.031
Trial No. 2					
Raw		Sl. feed	0	EtOH	0.156
Pretreated		Sl. feed	0	H_2O	0.000
Treated	210	Sl. cooked	1	H_2O	0.012
Homogenized-treated	210	Sl. cooked	1	H_2O	0.021
Trial No. 3					
Raw		Sl. feed	0	EtOH	0.156
Pretreated		Sl. feed	0	H_2O	0.004
Treated	210	Sl. cooked	1	H_2O	0.015
Homogenized-treated	210	Sl. cooked	1	H_2O	0.029

[a] Samples were coded and judged by the senior author.

[b] The color developed at 20° C. or lower was compared with a series of standards numbered from 0 to 6.

[c] The raw milks were titrated in 95% ethanol and the values indicate the total number of sulfhydryl groups capable of activation by heat treatment.

[d] The sulfhydryl values are expressed as milliequivalents of cysteine-HCl/liter.

had higher active sulfhydryl contents than the corresponding treated samples. Nevertheless, the total degree of activation was still low in all of the samples.

The observations of Boyd and Gould (3) may be pertinent to an explanation of these findings. They reported evidence that the production of volatile sulfhydryl compounds is a two-step process: first, the production of materials which reduce thiamine disulfide (excluding hydrogen sulfide); and secondly, the decomposition of a portion of these materials to permit evolution of hydrogen sulfide. They showed that thiamine disulfide values, computed as cysteine hydrochloride, were highest at the time the 90° C. (194° F.) temperature was achieved, ranging from 19-22 mg/liter (.121-.139 m.eq/liter), and became progressively less as heating and/or aspiration progressed. At any given temperature and time within the critical range of reactive sulfhydryl production, it appeared that hydrogen sulfide was produced rather rapidly, and the production corresponded to a loss in thiamine disulfide values. Since the milk was held at a high temperature for only a few seconds in the steam-vacuum unit, little time was available for the production of either volatile sulfhydryls or hydrogen sulfide. Of course, some of the volatile sulfhydryls or hydrogen sulfide may have been removed in the vacuum chambers of the steam-vacuum unit.

The observation that the milk treated in the steam-vacuum unit had a cooked flavor but a very weak nitroprusside test is contrary to the reported literature. One might question, therefore, the contribution of the volatile sulfhydryls to the cooked flavor in milk. These results indicate that the amount of volatile sulfhydryls necessary for a cooked flavor in milk may be less than that which is

actually necessary to yield a positive nitroprusside test; or perhaps all that is necessary for cooked flavor development is mere sulfhydryl activation. A third possibility is that there are substances other than sulfhydryl compounds which contribute towards cooked flavor in milk.

Effect of treatment on oxygen and ascorbic acid content. The results of the initial experiment to determine the effect of processing on the oxygen and ascorbic acid content of milk are shown in Table 5. In this experiment, steam

TABLE 5

Oxygen and ascorbic acid contents of raw, regular, homogenized, and homogenized-treated milks observed over a 96-hr. period [a]

Sample	Oxygen [b]	Ascorbic Acid [c]
	(m.eq/liter)	
Raw		
Fresh	1.081	0.116
24 hr.	0.890	0.081
48 hr.	0.773	0.050
72 hr.	0.679	0.030
96 hr.	0.518	0.015
Regular		
Fresh	1.178	0.123
24 hr.	1.214	0.090
48 hr.	1.114	0.068
72 hr.	1.086	0.036
96 hr.	1.029	0.018
Homogenized		
Fresh	1.279	0.123
24 hr.	1.391	0.111
48 hr.	1.296	0.091
72 hr.	1.144	0.077
96 hr.	1.164	0.039
Homogenized-treated [d]		
Fresh	0.410	0.125
24 hr.	0.459	0.131
48 hr.	0.498	0.131
72 hr.	0.436	0.129
96 hr.	0.533	0.125

[a] Samples were stored in the dark at 40° F.
[b] The oxygen values represent the averages of values obtained in two separate trials.
[c] The ascorbic acid values represent the averages of values obtained in six separate trials.
[d] The homogenized-treated samples were heated to 190° F. by infusing steam into the first chamber of the steam-vacuum unit.

was infused into the first chamber of the steam-vacuum unit. As may be seen, the initial oxygen content was much lower in the homogenized-treated milk than in the raw, regular, or homogenized milk. Over a 96-hr. period, the oxygen content increased slightly in the homogenized-treated milk, whereas it decreased in the other milks. The large decrease in the raw milk was probably due to bacterial action.

The initial ascorbic acid content was about the same for all samples, as may be seen in Table 5. There was no appreciable change in the ascorbic acid content of the homogenized-treated milk over the 96-hr. period. The other milks showed

very marked losses in ascorbic acid, but the homogenized milk showed more resistance to ascorbic acid oxidation than did the raw and regular milks. In comparing the oxygen and ascorbic acid content of the regular versus the homogenized milk, it is obvious that, in this case, the oxygen level was not the critical factor in determining the relative rates of ascorbic acid destruction. However, in the case of the homogenized versus the homogenized-treated milk, it would seem reasonable to assume that the low oxygen level in the latter milk contributed to the stability of the ascorbic acid.

Since the trend is towards longer storage of milk between processing and consumption, it was decided to measure ascorbic acid retention over a period of 2 wk. Furthermore, to pinpoint the effects of the various steps in the process, samples of pretreated, treated, and homogenized-treated milks were obtained. It should be pointed out that the homogenized-treated milk used in this experiment had been heated to 210 ± 2° F. in the U.H.T. heater, whereas in the previous experiment it had been heated to 190 ± 2° F. in the first chamber of the steam-vacuum unit.

TABLE 6

Ascorbic acid contents of pretreated, treated, and homogenized-treated milks observed over a 2-wk. period [a]

Sample	Ascorbic Acid *(m.eq/liter)*			Retention *(%)*	
	Fresh	1 wk.	2 wk.	1 wk.	2 wk.
Pretreated					
Trial 1	0.128	0.037	0.016	28.9	12.5
Trial 2	0.126	0.048	0.023	38.1	18.2
Treated [b]					
Trial 1	0.128	0.106	0.024	82.9	18.7
Trial 2	0.134	0.128	0.114	95.6	85.1
Homogenized-treated [b]					
Trial 1	0.128	0.124	0.079	97.0	61.7
Trial 2	0.134	0.136	0.136	101.3	101.3

[a] Samples were stored in the dark at 40° F.
[b] The treated samples were heated to 210° F. in the U.H.T. heater.

The results of this experiment are shown in Table 6. Although there were considerable differences between the rates of destruction of ascorbic acid in the two trials, the general trends were the same in both cases. All of the fresh samples had approximately the same ascorbic acid concentration but, by the end of a week, it had dropped drastically in the pretreated milk, whereas the treated and homogenized-treated samples showed very little change. Additional losses occurred during the second week, with the pretreated milk showing the greatest losses and the homogenized-treated milk showing the least effect.

It seems logical to assume that the increased stability of the ascorbic acid in the treated and homogenized-treated samples, as contrasted to the pretreated samples, was due in part to the removal of oxygen in the steam-vacuum unit. However, Sharp *et al.* (29) concluded that it is necessary to lower the oxygen

content down to the range of 0.4 to 0.5 mg/liter (0.050 to 0.062 m.eq/liter) to prevent the rapid disappearance of ascorbic acid in pasteurized milk over a 1-wk. period. Therefore, it would seem reasonable to conclude that the lowered oxygen content was not solely responsible for the high retention of ascorbic acid.

Undoubtedly, the higher heat treatment received by the treated and homogenized-treated samples contributed to the greater ascorbic acid stability. The literature reveals that the high heat treatment will stabilize ascorbic acid, at least for periods up to 72 hr. (3). Josephson and Doan (17) believed that the stability over a 48-hr. period was due to sulfhydryl groups released on heating milk to 170° F. or over. Gjessing and Trout (9) reported that the critical flash (15-sec.) exposure minima for ascorbic acid stability were between 75° C. (167° F.) and 85° C. (185° F.). Woessner *et al.* (36) concluded that the advantage of the flash pasteurization process in preserving the vitamin C content of milk could be attributed to the fact that the destruction of the ascorbic acid appears to be more dependent upon the time that it is held at an elevated temperature than on the temperature itself. Apparently, the short period of time that milk was held at a high temperature in the steam-vacuum unit was sufficient to contribute to the ascorbic acid stability. This conclusion is supported by the fact that the treatments producing increased stability also resulted in increased sulfhydryl activation (see Table 2).

To determine the magnitude of the effect of oxygen concentration on ascorbic acid stability, a sample of homogenized-treated milk was oxygenated. The determination of the total vitamin C retention over a 2-wk. period was a secondary purpose of this study.

The results of this experiment are shown in Table 7. A comparison of either the total or reduced ascorbic acid values for homogenized-treated and oxygenated homogenized-treated milks having the same heat treatment showed that the retention was nearly the same over the first 96 hr. Subsequently, the vitamin C in the oxygenated samples was slowly depleted and after 2 wk. it was almost entirely oxidized. It may be that the increased oxygen content of the milk promoted more rapid oxidation of sulfhydryl groups, so that after 96 hr. they were depleted, and then the oxidation of ascorbic acid proceeded at a more rapid rate. A comparison of the rate of oxidation of reduced ascorbic acid in the treated milk (Table 6) with that of the oxygenated homogenized-treated milk (Table 7) provides indirect evidence of the role of sulfhydryl compounds in ascorbic acid oxidation. On the basis of the oxygen values reported in Tables 5 and 7, it is assumed that the pretreated milk had a lower oxygen concentration than the oxygenated homogenized-treated milk; yet, during the first week the ascorbic acid was more rapidly oxidized in the former milk. However, by the end of 2 wk. the ascorbic acids levels were about the same in these two milks. This supports the theory that sulfhydryl groups merely act to prolong the induction phase of oxidation.

The retention of total vitamin C in the homogenized-treated milk over a 2-wk. period was found to be around 90%. The additional 20° F. heat treatment

TABLE 7

Vitamin C contents of homogenized-treated and oxygenated homogenized-treated milks observed over a 2-wk. period [a]

Sample	Oxygen	Vitamin C		
		Total	Reduced	Dehydro
		(m.eq/liter)		
Homogenized-treated, 210° F.				
Fresh		0.157	0.141	0.016
24 hr.		0.157	0.130	0.027
48 hr.	0.561	0.179	0.137	0.042
96 hr.		0.157	0.135	0.022
120 hr.		0.152	0.128	0.024
1 wk.		0.145	0.122	0.023
2 wk.		0.140	0.115	0.025
Oxygenated-homogenized-treated, 210° F.				
Fresh		0.157	0.130	0.027
24 hr.		0.153	0.130	0.023
48 hr.	1.951	0.171	0.122	0.049
96 hr.		0.149	0.121	0.028
120 hr.		0.116	0.096	0.020
1 wk.		0.106	0.063	0.043
2 wk.		0.025	0.014	0.011
Homogenized-treated, 230° F.				
Fresh		0.160	0.134	0.026
24 hr.		0.157	0.130	0.027
48 hr.	0.476	0.167	0.133	0.034
96 hr.		0.157	0.139	0.018
120 hr.		0.148	0.124	0.024
1 wk.		0.149	0.118	0.031
2 wk.		0.143	0.115	0.028
Oxygenated-homogenized-treated, 230° F.				
Fresh		0.157	0.130	0.027
24 hr.		0.153	0.130	0.023
48 hr.	2.109	0.175	0.129	0.046
96 hr.		0.142	0.124	0.018
120 hr.		0.136	0.112	0.024
1 wk.		0.133	0.102	0.031
2 wk.		0.029	0.014	0.015

[a] Samples were stored in the dark at 40° F.

received by the 230° F. milk, as compared to the 210° F. milk, did not appear to have any effect on vitamin C retention.

A comparison of the rate of ascorbic acid oxidation and rate of oxidized flavor development. The observations of several workers (5, 12, 13, 22, 30) have indicated a positive correlation between these two rates. Experiments were conducted to determine if this relationship was the same in regular, homogenized, and homogenized-treated milk. Inasmuch as the latter had shown considerable resistance to the ascorbic acid oxidation, copper was added to accelerate the oxidation. In the first trial, copper was added to give concentrations of 0.2 and 0.5 p.p.m., but since this did not have any appreciable effect on oxidized flavor development in the homogenized-treated milk, a second trial was run using 2.5 and 5.0 p.p.m. of copper. The results obtained for the first 96 hr. are shown in Table 8. The recorded flavor judgments are the average of the scores given to the milks by three judges in the first trial and four judges in the second trial.

TABLE 8

Comparison of the oxidation of ascorbic acid and oxidized flavor development in regular, homogenized, and homogenized-treated milks containing various levels of copper

Trial No.	Sample	Amount of Cu	Ascorbic Acid (m.eq/liter)				Oxidized flavor development [a]			
			Time in hours				Time in hours			
			Fresh	24	48	96	Fresh	24	48	96
		(p.p.m.)								
1	Regular	0.0	0.119	0.027	0.015	0.019	0	0	0	0
2		0.0	0.120	0.065	0.027	.....	0	0	?	0
1		0.2	0.119	0.000	0.000	.....	0	0	1	1
1		0.5	0.119	0.000	0.000	.....	?	1	2	3
2		2.5	0.120	0.000	0.000	.....	?	3	4	4
2		5.0	0.120	0.000	0.000	.....	0	4	4	4
1	Homogenized	0.0	0.126	0.096	0.073	0.031	0	0	0	0
2		0.0	0.120	0.092	0.081	.....	0	0	0	0
1		0.2	0.126	0.008	0.004	0.000	0	0	0	0
1		0.5	0.126	0.008	0.004	0.000	0	0	0	0
2		2.5	0.120	0.012	0.000	.....	?	2	3	3
2		5.0	0.120	0.008	0.000	.....	?	3	4	4
1	Homogenized-treated, 200° F.	0.0	0.126	0.123	0.123	0.117	0	0	0	0
2		0.0	0.120	0.115	0.117	.....	0	0	0	0
1		0.2	0.126	0.027	0.008	0.000	0	0	0	0
1		0.5	0.126	0.027	0.004	0.000	0	?	0	0
2		2.5	0.120	0.012	0.000	.....	0	0	2	3
2		5.0	0.120	0.008	0.000	.....	0	?	2	4
1	Homogenized-treated, 230° F.	0.0	0.126	0.119	0.123	0.121	0	0	0	0
2		0.0	0.120	0.119	0.117	.....	0	0	0	0
1		0.2	0.126	0.073	0.019	0.000	0	0	0	0
1		0.5	0.129	0.023	0.012	0.000	0	0	0	0
2		2.5	0.120	0.008	0.000	.....	0	0	2	4
2		5.0	0.120	0.008	0.000	.....	0	?	?	4

[a] Scoring system: 0, No oxidized flavor; ?, oxidized flavor doubtful; 1, slight oxidized flavor; 2, definite oxidized flavor; 3, strong oxidized flavor; and 4, very strong oxidized flavor.

As was expected, the regular milk showed more rapid deterioration of ascorbic acid and flavor than either the homogenized or homogenized-treated milk. The homogenized-treated milks containing 2.5 and 5.0 p.p.m. of added copper showed more resistance to oxidized flavor development than the corresponding homogenized milk. However, the rates of ascorbic acid oxidation were similar in these milks. At the 0.2 and 0.5 p.p.m. levels of added copper, homogenized, homogenized-treated at 200° F., and homogenized-treated at 230° F. showed increasing resistance to ascorbic acid oxidation, in this order. None of these three milks showed any development of oxidized flavor even after 14 days of storage. Although these findings confirm the general relationship between ascorbic acid oxidation and oxidized flavor development, they indicate that in the homogenized products, as contrasted to the unhomogenized products, copper has a greater effect on the ascorbic acid oxidation than on oxidized flavor development.

Although this study did not involve a comparison of the flavor stability of treated with homogenized-treated milk, a previous study by Shipe (31) revealed

that neither milk showed any oxidized flavor over a 2-wk. period. Therefore, one should not assume that homogenization was the major factor in the flavor stability of the homogenized-treated milk.

REFERENCES

(1) Aibara, K., Herreid, E. O., and Wilson, H. K. Effect of the Structure of Some Mercaptans on Their Amperometric Titration with Silver Nitrate. J. Dairy Sci., 43: 1736. 1960.

(2) Benesch, R. E., Lardy, H. A., and Benesch, R. The Sulfhydryl Groups of Crystalline Proteins. I. Some Albumins, Enzymes, and Hemoglobins. J. Biol. Chem., 216: 663. 1955.

(3) Boyd, E. N., and Gould, I. A. Volatile and Nonvolatile Sulfhydryl Content of Heated Milk and Milk Proteins. J. Dairy Sci., 40: 1294. 1957.

(4) Burton, H. The Amperometric Titration of Sulfhydryl Groups at a Rotating Platinum Electrode. Biochim. et Biophys. Acta, 29: 193. 1958.

(5) Chilson, W. H., Martin, W. H., and Parrish, D. B. The Relationship of Ascorbic Acid to the Development of Oxidized Flavor in Market Milk. J. Dairy Sci., 32: 306. 1949.

(6) Dahle, C. D., and Palmer, R. G. The Oxidized Flavor in Milk from the Individual Cow. Pennsylvania Agr. Expt. Sta., Bull. 347. 1937.

(7) Emmons, D. B., Swanson, A. M., and Price, W. V. Effect of Skimmilk Heat Treatments on Cottage Cheese Manufacture. J. Dairy Sci., 42: 1020. 1959.

(8) Forster, R. L., and Sommer, H. H. Manganese, Trypsin, Milk Proteins and the Susceptibility of Milk to Oxidized Flavor Development. J. Dairy Sci., 34: 992. 1951.

(9) Gjessing, E. C., and Trout, G. M. Ascorbic Acid and Oxidized Flavor. II. The Effect of Various Heat Treatments of Milk on the Stability of Ascorbic Acid and on the Development of Oxidized Flavor. J. Dairy Sci., 23: 373. 1940.

(10) Gunsalus, I. C., and Hand, D. B. The Use of Bacteria in the Chemical Determination of Total Vitamin C. J. Biol. Chem., 141: 853. 1941.

(11) Harland, H. A., and Ashworth, U. S. The Preparation and Effect of Heat Treatment on the Whey Proteins of Milk. J. Dairy Sci., 28: 879. 1945.

(12) Hartman, G. H., and Garrett, O. F. Some Factors Affecting the Stability of Certain Milk Properties. VI. Relation of the Concentration of Dissolved Oxygen to the Development of Oxidized Flavor in Milk. J. Dairy Sci., 26: 337. 1943.

(13) Henderson, J. L. The Vitamin C Content of Milk and Its Relation to Oxidized Flavor. Intnl. Assoc. Milk Dealers, Assoc. Bull., 12: 271. 1940.

(14) Herrington, B. L. Personal communication. Cornell University. 1959.

(15) Hutton, J. T., and Patton, S. The Origin of Sulfhydryl Groups in Milk Proteins and Their Contribution to "Cooked" Flavor. J. Dairy Sci., 35: 699. 1952.

(16) Jenness, R. Milk Proteins: Effects of Heat Treatment on Serum Proteins. J. Agr. and Food Chem., 2: 75. 1954.

(17) Josephson, D. V., and Doan, F. J. Observations on Cooked Flavor in Milk—Its Source and Significance. The Milk Dealer, 29 (2): 35. 1939.

(18) Kolthoff, I. M., and Harris, W. E. Amperometric Titration of Mercaptans with $AgNO_3$ Using the Rotating Platinum Electrode. Ind. Eng. Chem., Anal. Ed., 18: 161. 1946.

(19) Kolthoff, I. M., Streets, W., and Morren, Lois. Amperometric-Mercurimetric Titration of Sulfhydryl Groups in Biologically Important Substances at the Rotated Platinum Wire Electrode as Indicator Electrode. Anal. Chem., 26: 366. 1954.

(20) Ma, T. S., and Zuazaga, G. Micro-Kjeldahl Determination of Nitrogen. Ind. Eng. Chem., Anal. Ed., 14: 280. 1942.

(21) Menefee, S. G., Overman, O. R., and Tracy, P. H. The Effect of Processing on the Nitrogen Distribution in Milk. J. Dairy Sci., 24: 953. 1941.

(22) Nelson, D. H., and Dahle, C. D. Enzymes and Other Substances as Anti-oxidants in Milk. The Milk Dealer, 29: 41. 1940.

(23) Parks, T. D., and Lykken, L. Determination of Small Amounts of Silver in Lubricating Oils by Amperometric and Gravimetric Methods. Anal. Chem., 22: 1505. 1950.

(24) Patton, S., and Josephson, D. V. Observations on the Application of the Nitroprusside Test to Heated Milk. J. Dairy Sci., 32: 398. 1949.

(25) Peterson, R. D. Determination of the Time Differential Between the Fastest and Slowest Particles Flowing Through a UHT and a Combined UHT-HTST Pasteurization Unit. Master's thesis. Cornell University. 1960.

(26) Rowland, S. J. The Determination of the Nitrogen Distribution in Milk. J. Dairy Research, 9: 42. 1938.

(27) Shahani, K. M., and Sommer, H. H. The Protein and Nonprotein Nitrogen Fraction in Milk. III. The Effects of Heat Treatments and Homogenization. J. Dairy Sci., 34: 1035. 1951.

(28) Sharp, P. F., Hand, D. B., and Guthrie, E. S. Quantitative Determination of Dissolved Oxygen. Ind. Eng. Chem., Anal. Ed., 13: 593. 1941.

(29) Sharp, P. F., Hand, D. B., and Guthrie, E. S. Experimental Work on Deaeration of Milk. A paper presented at Lab. Sec. of 34th Ann. Conv. of Intnl. Assoc. Milk Dealers, Toronto, Ontario. 1941.

(30) Sharp, P. F., Trout, G. M., and Guthrie, E. S. Vitamin C, Copper, and the Oxidized Flavor in Milk. 10th Ann. Rept. New York State Assoc. Dairy and Milk Inspectors. 1936.

(31) Shipe, W. F., Jr. The Effect of Steam Injection and Vacuum Treatment on the Flavor of Milk. J. Dairy Sci., 42: 895. 1959.

(32) Sluyterman, L. A. The Amperometric Titration of Sulfhydryl Groups with Silver Nitrate. Biochim. et Biophys. Acta, 25: 402. 1957.

(33) Stewart, A. P., Jr., and Sharp, P. F. Determination of Vitamin C in the Presence of Interfering Reducing Substances. Ind. Eng. Chem., Anal. Ed., 17: 373. 1945.

(34) Tarassuk, N. P., and Koops, J. Inhibition of Oxidized Flavor in Homogenized Milk as Related to the Concentration of Copper and Phospholipids per Unit of Fat Globule Surface. J. Dairy Sci., 43: 93. 1960.

(35) Townley, R. C., and Gould, I. A. A Quantitative Study of the Heat Labile Sulfides of Milk. II. General Origin of Sulfides and Relation to Total Sulfur, Total Nitrogen, and Albumin Nitrogen. J. Dairy Sci., 26: 843. 1943.

(36) Woessner, W. W., Weckel, K. G., and Schuette, H. A. The Effect of Commercial Practices on Ascorbic Acid and Dehydroascorbic Acid (Vitamin C) in Milk. J. Dairy Sci., 23: 1131. 1940.

CRYSTALLINE RENNIN IN THE MANUFACTURE OF CHEDDAR CHEESE [1, 2]

P. M. LINKLATER [3] AND C. A. ERNSTROM
Department of Dairy and Food Industries, University of Wisconsin, Madison

SUMMARY

Unsuccessful attempts to produce good Cheddar cheese with crystalline rennin led other workers to suggest the cheese-making value of substances other than rennin in rennet extract. Eight three-vat lots of Cheddar cheese were made to compare the effects of crystalline rennin, commercial rennet, and the supernatant resulting from the salt precipitation of about 80% of the clotting activity from rennet extract. The activity of each rennin preparation was measured in a reconstituted substrate (60 g. nonfat dry milk in 500 ml. 0.01 *M* $CaCl_2$). Clotting solutions containing equal substrate clotting activities were added to each vat. The cheese, cured at 50° F., was of good quality, and no significant differences were apparent between treatments when graded organoleptically at 2, 10, and 34 wk. Small but consistent differences in soluble nitrogen content were noted. Cheese made with crystalline rennin yielded the highest soluble nitrogen, and that made with the supernatant the lowest.

Good-quality Cheddar cheese was produced with crystalline rennin. Other substances in rennet extract were found to be nonessential to the cheese-making process.

Berridge (2) reported that milk treated with crystalline rennin clotted quickly, but that the curd remained soft for relatively long periods. This curd, when manufactured into cheese, lacked flavor and was rather unpleasant at 4 mo. of age. Impurities discarded in the preparation of crystalline rennin were also used in cheese manufacture, and caused an intensification of the normal flavor of Cheddar cheese. Berridge (3) reported that on repetition and extension of this work the results were inconclusive, possibly because the control cheese was poor in quality. However, attention was drawn to the significant fact that good cheese had never been made with crystalline rennen. The implication was that some component of rennet, other than rennin, was essential for the manufacture of good-quality Cheddar cheese.

The object of this study was to determine whether good-quality Cheddar cheese could be made with crystalline rennin, to compare the strength of curds formed with crystalline rennin and rennet extract, and to investigate the possibility that nonrennin material in rennet extract contributes to the quality of Cheddar cheese.

EXPERIMENTAL METHODS

On eight different occasions between February and September, 1958, three vats of Cheddar cheese were made for the purpose of comparing the be-

Received for publication April 10, 1961.

[1] Published with the approval of the Director, Wisconsin Agricultural Experiment Station.

[2] This study was supported in part by a research grant from Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin.

[3] Permanent address—South Australian Department of Agriculture, Adelaide, Australia.

havior of crystalline rennin, commercial rennet, and the supernatant solution remaining after removal of most of the rennin from commercial rennet.

Milk clotting solutions. The preparation of crystalline rennin has been described (7). In this procedure the first step was to adjust commercial rennet to pH 5.0 and saturate with NaCl to precipitate the rennin. The supernatant liquid contained about one-fifth the clotting activity of the original rennet and approximately four times the proteinaceous material per unit of milk clotting activity. Hereafter, this solution will be referred to as the supernatant. Fresh commercial rennet served as a control treatment. The strengths of the commercial rennet,[4] the supernatant, and the crystalline rennin solutions were calculated from their milk-clotting activities in Berridge's (1) substrate prepared by dissolving 60 g. nonfat dry milk (NDM) in 500 ml. of 0.01 *M* $CaCl_2$. On the basis of their activities in the special substrate, each rennin preparation used in the experimental treatments was equivalent in activity to 3 oz. of commercial rennet per 1,000 lb. of milk.

Cheese manufacture. Milk was obtained from the mixed Grade A supply of the University of Wisconsin Dairy Manufacturing Laboratory, pasteurized at 161° F. for 16 sec., and stored overnight at 38° F. before making it into cheese. The average fat content of the milk was 3.5%. Cheese was made in three 500-lb. experimental vats using 230 lb. of milk and 2.3 lb. of lactic starter per vat. The three clotting solutions were diluted tenfold with cold water before they were added to the cheese milk. A general manufacturing procedure (11) was followed, except where minor changes in timing were required by fluctuations in starter activity. The cheese was pressed into 20-lb. blocks, cut into 5-lb. loaves, wrapped in Parakote, and cured at 50° F. for 34 wk.

Curd strength. The pasteurized cheese milk was sampled for curd-strength measurements each day prior to cheese manufacture. Dilutions of crystalline rennin and supernatant were standardized in Berridge's (1) substrate to contain the same activity per milliliter as a 1:50 dilution of commercial rennet. Two hundred milliliters of milk were pipetted into a 250-ml. beaker, and equiliberated for half an hour in a water bath at 30° C. Two milliliters of the appropriately diluted clotting solution were thoroughly mixed into the milk. Eight beakers of milk were thus prepared for each treatment, to give curd strength measurements at 20, 25, 30, 35, 40, 45, 50, and 55 min. after addition of the clotting solution. Curd strength was measured with a Cherry-Burrell curd tension meter.

Cheese analysis. Moisture content of the cheese at 2 wk. of age was determined in duplicate by the method of Price *et al.* (12). The cheese was sampled at 10 and 34 wk. of age for extraction of soluble nitrogen by the method of Vakaleris and Price (14). Nitrogen determinations were made on the extract and on the cheese by the micro-Kjeldahl procedure of Hiller, Plazin, and Van Slyke (9). The soluble nitrogen was expressed as per cent of total nitrogen.

The cheese was graded at 2, 10, and 34 wk. by three judges for flavor, body, and total score in accordance with the following scale: 1—Excellent; 2—De-

[4] Supplied by Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin.

sirable; 3—Satisfactory; 4—Objectionable; 5—Very objectionable; 6—Unsalable. The experimental cheese was scored once by each judge at each period, and the individual scores averaged to obtain the score of the cheese.

RESULTS

Curd strength. Figure 1 shows a plot of average curd strength against time for eight replicates. Contrary to Berridge's (2) observation, crystalline rennin

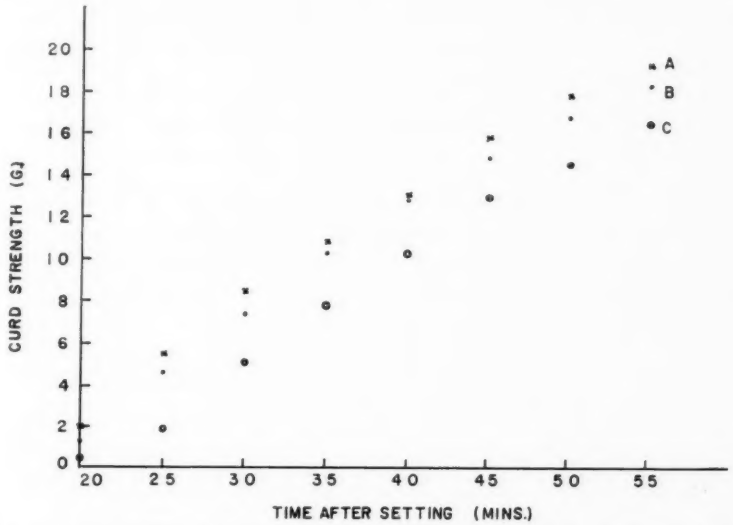


FIG. 1. Average curd strength of cheese milk coagulated by three rennet fractions.
A. Commercial rennet.
B. Crystalline rennin.
C. The supernatant solution prepared by adjusting commercial rennet to pH 5.0 and saturating with NaCl.

produced a satisfactory increase in curd strength. It was concluded that no adjustment in the cheese-making process would be necessary in order to use the purified enzyme. Differences in strength of curd formed with crystalline rennin and curd formed with commercial rennet were not statistically significant. It appeared that commercial rennet used in these experiments contained no unidentified factor essential for the production of a firm coagulum. The supernatant solution consistently produced curd with the lowest average curd strength. This solution raised the NaCl concentration in the milk by 0.04%, which may have had a depressing effect on the curd strength (13), but it is unlikely that the amount of salt present could reduce the curd strength as much as was observed in this experiment.

Cheese quality. Results presented in Table 1 show that the average flavor scores of all cheese fell between satisfactory and desirable. The maximum differ-

TABLE 1

Average scores[a] of cheese made with three different rennet fractions

	Treatment	Age of cheese		
		2 wk.	10 wk.	34 wk.
Flavor score	Crystalline rennin	2.48	2.63	2.74
	Commercial rennet	2.44	2.74	2.72
	Supernatant	2.57	2.60	2.56
Body score	Crystallin rennin	2.94	2.38	2.24
	Commercial rennet	3.02	2.49	2.29
	Supernatant	3.11	2.61	2.23
Total score	Crystalline rennin	2.72	2.57	2.66
	Commercial rennet	2.78	2.80	2.68
	Supernatant	2.80	2.70	2.48

[a] 1—excellent; 2—desirable; 3—satisfactory; 4—objectionable; 5—very objectionable; and 6—unsalable.

ence between treatments at any one grading was 0.18 point. Flavor scores at 2, 10, and 34 wk. showed no consistent differences in the cheese made with the three coagulants. There was no sign of the flavor differences reported by Berridge (2). A similar lack of consistent differences was evident in the body and total scores.

Cheese analysis. The average moisture content of the cheese made with commercial rennet, crystalline rennin, and the supernatant was 36.6, 37.0, and 36.6%, respectively, at 2 wk. of age.

The ripening of the cheese was followed by soluble nitrogen determinations (14), which provided an objective measure of protein breakdown. Results of the nitrogen analysis in Table 2 show the soluble nitrogen expressed as per cent of total cheese nitrogen.

TABLE 2

Effect of three different rennet fractions on the soluble nitrogen[a] in cheese at 10 and 34 wk.

Treatment	Soluble nitrogen[a]	
	10 wk.	34 wk.
	(%)	
Crystalline rennin	19.7	31.5
Commercial rennet	18.7	30.1
Supernatant	17.1	27.4

[a] Soluble nitrogen as per cent of total nitrogen.

Soluble nitrogen was highest in cheese made with crystalline rennin, followed by that made with commercial rennet, and lowest in cheese made with the supernatant solution. This order was maintained in every case except one where the commercial-rennet cheese was slightly higher in soluble nitrogen than the crystalline-rennin cheese at both 10 and 34 wk.

An analysis of variance and Duncan's (5) multiple range test revealed that all differences between mean values in Table 2 for 10-wk.-old cheese were significant at the 1% level of probability. At 34 wk., differences in soluble nitrogen

between cheese made with supernatant and that made with commercial rennet and crystalline rennin were significant at the 5 and 1% levels, respectively. At the same age the difference in soluble nitrogen between cheese made with crystalline rennin and that made with commercial rennet was not significant.

DISCUSSION

Differences in soluble nitrogen in cheese made with the three clotting solutions could not be attributed to any obvious differences between these solutions. The nitrogen differences were small but interesting, because they were of the same order as those obtained when cheese was made with normal and half-normal amounts of rennet (8). Therefore, the method of determining the volume of these clotting solutions required for cheese-making was investigated (10). In these experiments the strength of the crystalline rennin and supernatant in Berridge's (1) substrate was calculated in rennin units per milliliter, with commercial rennet as the standard (7). On this basis, an equal number of rennin units was used in each vat of cheese. An ideal basis would have been measurement of rennin activity in skimmilk, because it has nearly the same clotting characteristics as cheese milk. In practice this is impracticable, because the clotting time of skimmilk is not reproducible. However, tests conducted in skimmilk showed that fewer rennin units from commercial rennet than crystalline rennin were added to the cheese milk. The amount of commercial rennet required for a true comparison was slightly underestimated. This was attributed to pepsin in the commercial rennet which had greater activity when tested in Berridge's substrate than when used in the cheese vat (10). This difference in activity was not reflected in curd strength measurements or in soluble nitrogen in 34-wk.-old cheese, where differences between the crystalline rennin and commercial rennet treatments were not statistically significant. However, in younger cheese the difference in soluble nitrogen between these two treatments was highly significant.

Pepsin is soluble in saturated NaCl (6), and was selectively concentrated in the supernatant. Therefore, the larger amount of pepsin caused an even greater underestimate of the amount of supernatant required in these experiments. This underestimate contributed in part to the significantly lower curd strength and soluble nitrogen values in cheese made with this solution.

Data taken from the study of Davies *et al.* (4) gave a comparison of the breakdown of cheese protein by pepsin and rennin. The experimental lots of cheese were made in pairs on three occasions; one of each pair was made with pepsin, the other with rennin. In every case the nonprotein nitrogen, expressed as per cent of total nitrogen, was higher in cheese made with rennet than that made with pepsin. The average values were 27.0% in rennet cheese and 25.4% in pepsin cheese. These results, though not conclusive, suggest that rennin is more active than pepsin in breaking down cheese protein. This suggests an explanation for the greater proteolytic activity of the crystalline rennin in the present experiments. The commercial rennet and supernatant contained some

pepsin, which could account for the lower soluble nitrogen values in the cheese where these solutions were used.

The differences in soluble nitrogen, shown in Table 2, did not affect the grade results shown in Table 1. It is clear that good-quality cheese was made with crystalline rennin, and that quality was unaffected when four times the normal level of nonrennin material from commercial rennet was used in cheese manufacture.

It is concluded that rennin is the only essential component of commercial rennet when it is used to manufacture Cheddar cheese under conditions comparable with this experiment.

REFERENCES

(1) Berridge, N. J. Some Observations on the Determination of the Activity of Rennet. Analyst, 77: 57. 1952.

(2) Berridge, N. J. Preparation of a Cheese with Crystalline Rennin. J. Dairy Research, 22: 384. 1955.

(3) Berridge, N. J. Crystalline versus Crude Rennet in Cheese Making. Intern. Dairy Congr. Proc., 2: (2) 59. 1956.

(4) Davies, W. L., Davis, J. G., Dearden, D. V., and Mattick, A. T. R. Studies in Cheddar Cheese. III. The Role of Rennin, Pepsin, and Lactobacilli. J. Dairy Research, 5: 144. 1934.

(5) Duncan, D. B. Multiple Range and Multiple F Tests. Biometrics, 11: 1. 1955.

(6) Effront, J. Biochemical Catalysis in Life and Industry. John Wiley and Sons, Inc., New York. 1917.

(7) Ernstrom, C. A. Heterogeneity of Crystalline Rennin. J. Dairy Sci., 41: 1663. 1958.

(8) Ernstrom, C. A., Price, W. V., and Swanson, A. M. Effects of Reducing Rennet and Adding Calcium Chloride on the Manufacture and Curing of Cheddar Cheese. J. Dairy Sci., 41: 61. 1958.

(9) Hiller, A., Plazin, J., and Van Slyke, D. D. A Study of Conditions for Kjeldahl Determination of Nitrogen in Proteins. J. Biol. Chem., 176: 1401. 1948.

(10) Linklater, P. M. The Significance of Rennin and Pepsin in Rennet. Ph.D. thesis, University of Wisconsin. 1961.

(11) Price, W. V., and Calbert, H. C. Cheddar Cheese from Pasteurized Milk. Wisconsin Agr. Expt. Sta., Bull. 464. 1944. (Rev. 1953).

(12) Price, W. V., Winder, W. C., Swanson, A. M., and Sommer, H. H. The Sampling of Cheddar Cheese for Routine Analysis. J. Assoc. Offic. Agr. Chemists, 36: 524. 1953.

(13) Sirry, I., and Shipe, W. F. Effect of Sodium Chloride on the Curd Tension of Rennet-Coagulated Milk. J. Dairy Sci., 41: 204. 1958.

(14) Vakaleris, D. G., and Price, W. V. A Rapid Spectrophotometric Method for Measuring Cheese Ripening. J. Dairy Sci., 42: 264. 1959.

MODIFIED REDUCTION TEST TO PREDICT THE SHELF LIFE OF COTTAGE CHEESE [1]

L. G. HARMON, G. C. WALKER, AND L. D. McGILLIARD
Departments of Food Science and Dairy, Michigan State University, East Lansing

SUMMARY

One hundred and one commercial samples of Cottage cheese were subjected to a modified reduction test designed to measure the correlation between shelf life and the reduction time required for resazurin, methylene blue, and 2,3,5-triphenyl tetrazolium chloride (TTC). Prior to testing, the samples were neutralized to a pH of 6.5 ± 0.5, bile salts No. 3 (Difco) were added to partially inhibit the Gram-positive lactic organisms, and trypticase soy broth was added to stimulate the growth of the psychrophiles. The reduction times varied from 1.3 to 40 hr., depending on the quality of the cheese and the type of dye used. The reduction times in hours at 24° C., plotted against keeping quality in days, gave correlation coefficients as follows: resazurin pink, 0.80; resazurin white, 0.77; methylene blue, 0.75; and TTC 0.74. Reduction to resazurin pink gave the most rapid results as well as the highest correlation. The correlations between shelf life and initial psychrophile and total count were −0.67 and −0.78, respectively.

The test is useful for identifying Cottage cheese with a short shelf life. Nineteen of 20 samples which developed an organoleptically detectable defect within two days when stored at 10° C., reduced resazurin to pink in 7.75 hr. or less, and all samples requiring more than 23 hr. to reduce resazurin retained their original organoleptic quality for six or more days.

In recent years the dye reduction tests originally applied to milk have been subjected to various modifications and applied to numerous food products, including some in which psychrophiles are of major importance. Scott and Gillespie (8, 9) and Hirshmann and Lightbody (4) used the resazurin reduction test to evaluate the quality of liquid eggs and reported a general relationship between reduction time and the plate count. Walker *et al.* (11) and Mallmann *et al.* (7) reported that reduction time of broth used to swab dressed poultry was related to the sanitary condition under which the poultry was handled. Good correlation between resazurin reduction times and total organism counts were reported by Straka and Stokes (10), who examined precooked frozen meat pies, and Kereluk and Gunderson (5), who worked with frozen vegetables, fruits, and meats.

Greene and Jamison (3) performed resazurin reduction tests on skimmilk inoculated with varying numbers of *Pseudomonas fluorescens* and reported reduction times of 45 to 90 min. with organism concentrations of 10^8 per milliliter, and 5 hr. with populations of 10^5 per milliliter. LaGrange and Nelson (6) performed resazurin and triphenyl tetrazolium chloride (TTC) dye reduction tests on 25 samples of low-count raw milk, each of which was inoculated with a different psychrophilic organism and incubated 72 hr. at 5° C. At the end of the incubation period, and at the time the reduction tests were performed, the bacteria counts in the milks exceeded six million per milliliter when enu-

Received for publication February 27, 1961.

[1] Michigan Agricultural Experiment Station Journal Article No. 2774.

merated on plates incubated three days at 23° C. None of the organisms reduced either dye in 2.75 hr. when the reduction tests were incubated at 28° C. At 35.5° C., a number of these organisms reduced TTC more rapidly than resazurin, but rate of reduction was not proportional to bacteria count. This was attributed to the poor reducing ability of the psychrophiles.

Preliminary experimentation in the work reported herein showed that the time required for samples of Cottage cheese to reduce resazurin, methylene blue, and TTC dyes varied from 12 to 48 hr., and there was no apparent relationship between reduction time and keeping quality. Many lactic organisms are naturally present in Cottage cheese and manufacturers sometimes inoculate the creaming mixture with 0.5 to 2.0% lactic culture. The action of these organisms on the dyes prevents the existence of a consistently valid relationship between reduction time and keeping quality. To circumvent this problem a modified reduction test was developed which involved the incorporation of an inhibitor for the Gram-positive organisms, and a growth stimulant for the Gram-negative psychrophiles responsible for spoilage.

EXPERIMENTAL PROCEDURE

Procurement of samples. The samples of Cottage cheese used in this work were secured in 1- or 2-lb. packages directly from manufacturers, during the months of July and August. Most of the samples were obtained the day they were made; however, a few samples were selected which were four to eight days old and three samples were 13 to 19 days old. All samples were maintained at 4.5° C. or lower and tested within two days.

Preparation of samples. Fifty grams of cheese were weighed into sterile Waring blendor jars to which were added 5 ml. of 0.75 *N* NaOH and 45 ml. of trypticase soy broth containing 0.3% bile salts No. 3 (Difco). The contents of the jars were blended for 2 min. and samples removed for pH determination, reduction tests, and bacteria counts. The remainder of the cheese was incubated at 10° C. and examined daily for evidence of deterioration detectable organoleptically. It was desired to induce deterioration as soon as possible and at 10° C. the psychrophiles usually overgrow the lactic acid–producing organisms; whereas, at higher temperatures these organisms frequently produce sufficient acid to inhibit the psychrophiles. In the work reported herein the shelf life is defined as the length of time in days, after analyses were performed, during which the samples retained a normal flavor and odor, or is one day less than the time required for a perceptible defect to appear.

Analytical procedures. A Beckman model H-2 pH meter equipped with a glass electrode was used to determine the pH of the original cheese and the mixture after blending. (Duplicate samples measured with a quinhydrone electrode showed a maximum difference of 0.06 of a pH unit.)

The Waring blendor jars were sterilized by soaking for 1 hr. or more in a hypochlorite solution containing 500 p.p.m. chlorine. The jars were then thoroughly rinsed with 100 ml. of the sterile, buffered rinse solution described in Standard Methods (1).

The dyes used in performing the reduction tests were resazurin, methylene blue, and 2,3,5-triphenyl tetrazolium chloride (TTC) in concentrations of 1:20,000, 1:25,000, and 1:1,000, respectively. Ten milliliters of the blended cheese and 1 ml. of the dye were mixed in sterile screw-cap tubes and the contents incubated at 24° C. in a covered Precision water bath.

Total counts were performed on tryptone glucose yeast extract agar (TGYE) and Gram-negative psychrophiles were enumerated on violet red bile (VRB) agar. The plates were incubated for three days at 22° C. and the counts were corrected for the dilution involved when the cheese was blended.

RESULTS AND DISCUSSION

In the preparation of samples the NaOH was incorporated to increase the pH to a range favorable to the psychrophiles. Organisms in the *Pseudomonas, Achromobacter,* and *Flavobacterium* genera utilize glucose, but normally do not ferment lactose; therefore, trypticase soy broth containing 0.25% glucose and a buffering agent in the form of K_2HPO_4 was added to stimulate the growth of Gram-negative organisms and to buffer the mixture against a decrease in pH during the incubation period of the reduction test. The selection of bile salts No. 3 for the inhibition of Gram-positive organisms was based on the results of exploratory tests described below.

Evaluation of alkyl aryl sulfonate (nacconal) as an inhibitor of Gram-positive bacteria. Nacconal has been recommended (2) for the inhibition of Gram-positive organisms in plating media and in solutions used in dye reduction tests. Appropriately designed tests on 25 samples of Cottage cheese prepared as described under Procedure (bile salts omitted) indicated that 0.1% nacconal would not inhibit Gram-positive organisms in reduction tests or plate counts involving low dilutions. Also, nacconal in increments ranging from 0 to 0.5% was not effective in inhibiting *Streptococcus lactis* during reduction tests at 24° C. on inoculated skimmilk enriched with 2.0% phytone, 2.0% dextrose, and 0.5% yeast extract. There was evidence to indicate that nacconal reacted with one of the components of the milk or cheese and, thus, became ineffective as a Gram-positive inhibitor in plates of low dilution and in reduction tests.

Evaluation of bile salts No. 3 and formate-ricinoleate as inhibitors of Gram-positive bacteria. Ten blended cheese samples were prepared as described under Procedure with the following additives: (a) 1.0% sodium ricinoleate plus 5.0% sodium formate, (b) 0.15% bile salts No. 3, and (c) control with no inhibitor added.

When 0.1 ml. of an 18-hr. culture of *S. lactis* was inoculated into cheese containing formate-ricinoleate, the reduction times with resazurin dye at 24° C. varied from 6.5 to 8.25 hr. Corresponding samples similarly inoculated with *Pseudomonas fragi* were reduced in 5.0 to 6.5 hr. When cheese containing bile salts No. 3 was inoculated with *S. lactis* or *P. fragi* the reduction times varied from 6.5 to 8.0 hr. and 3.5 to 4.5 hr., respectively. Inoculation with both *S. lactis* and *P. fragi* did not decrease the reduction time below the time required by the *Pseudomonas* alone. Duplicate samples containing neither formate-ricinoleate

nor bile salts reduced in 1.75 to 3.0 hr. when inoculated with *S. lactis,* indicating that both of these materials were inhibiting the reducing ability of *S. lactis.* When three species of lactobacilli (*Lactobacillus brevis, Lactobacillus casei,* and two strains of *Lactobacillus plantarum*) were individually inoculated into sterile milk, the time required to reduce resazurin varied from 4.5 to 9.5 hr., and averaged 6.0 hr. When 0.15% bile salts No. 3 were added to the milk, the reduction time varied from 9.5 to 22.75 hr. and averaged 12.5 hr., indicating inhibition of the organisms.

Examination of commercial samples of Cottage cheese. After securing the above results, bile salts No. 3 were selected as an appropriate inhibitor of Gram-positive organisms. Analyses were performed on 101 samples of commercial Cottage cheese, a number deemed sufficient to render the results statistically reliable. At the time the samples were procured the pH of the samples varied from 4.55 to 5.55 and averaged 5.09. After they were blended the pH varied from 5.80 to 7.15 and averaged 6.51. The pH of all but seven of the samples was within the range of 6.0 to 7.0.

Shelf life, reduction time, and bacteria counts of commercial Cottage cheese. The data in Table 1 show that the shelf life of cheese stored at 10° C. varied from one to seven or more days beyond the date of analysis. The average and the range of time required for reduction by each of the different methods increased as the keeping quality increased, and also the average bacteria counts decreased as the keeping quality increased. The averages of the reduction times for the entire group of 101 samples were as follows: resazurin pink, 13.9 hr.; methylene blue, 15.4 hr.; TTC, 16.2 hr.; and resazurin white, 16.3 hr.

As the keeping quality increased, the averages of the reduction times and the bacteria counts showed uniform increases and decreases, respectively. However, within each group the variations from the average overlap into adjacent groups. For example, an individual sample of cheese requiring 7 hr. to reduce resazurin to pink and with a total count of 1,000,000 per gram on TGYE agar has a predictable shelf life of two or three days at 10° C.

In general, the reduction times in the work reported herein are longer than those reported by workers using other food products with similar or greater total organism populations (4, 8-11), and may be attributed to the presence of the Gram-positive inhibitor.

The fact that psychrophiles have long generation times (2) and weak reducing ability (6) restricts the utility of the reduction test when psychrophiles are the principal spoilage organisms. With a few minutes for initial preparation, the normal working day permits the observation of samples during an incubation period of 7 to 8 hr. In this work, 20 of the 101 samples tested (data on individual samples are not shown) had reduction times of 1.25 to 7.75 hr. Three of these 20 samples had a shelf life of only one day, 13 had a shelf life of two days, and four had a shelf life of three days. Fourteen, or 70%, of these 20 samples of cheese had total counts of 1,000,000 or more per milliliter.

Eighteen of the 101 samples had total counts of 1,000,000 or more per milliliter. All of these reduced resazurin to pink in less than 12 hr. and had a maximum shelf life of four days.

TABLE 1

Shelf life, reduction times, and bacteria populations in 101 samples of commercial Cottage cheese

Shelf life in days when held at 10° C.	No. of samples		Reduction time in hours at 24° C.				Log average and range of initial plate counts per gram of cheese	
			Resazurin				3 days at 22° C.	
			Pink	White	M.B.	TTC	VRB	TGYE
1	3	Average	2.7	3.7	3.1	3.8	580,000	>6,000,000
		Range	2.5-3.0	3.5-4.0	3.0-3.3	3.0-5.3	550,000->600,000	all >6,000,000
2	14	Average	6.4	8.4	7.5	8.3	28,000	1,400,000
		Range	1.3-10.0	1.8-14.0	1.8-10.5	3.0-13.5	94->600,000	320,000->6,000,000
3	19	Average	10.6	13.2	12.3	12.8	810	74,000
		Range	6.0-16.0	10.0-18.5	8.5-17.0	8.0-18.5	2->600,000	2,800->6,000,000
4	13	Average	12.3	14.8	13.6	14.1	3,100	51,000
		Range	9.0-18.0	10.0-21.0	9.0-21.0	10.0-18.0	22-300,000	2,800-1,100,000
5	24	Average	15.2	18.1	16.8	18.4	390	8,700
		Range	9.5-23.0	12.5-28.0	10.5-28.0	11.0-29.0	2-60,000	160-600,000
6	16	Average	18.8	21.1	21.1	21.4	49	2,800
		Range	12.5-33.0	14.0-36.0	12.5-40.0	13.0-40.0	<2-2,400	360-15,000
7 or more	12	Average	22.9	25.0	24.0	25.5	37	2,300
		Range	15.0-33.0	16.0-36.0	15.0-35.0	15.0-36.0	<2-2,800	110-28,000

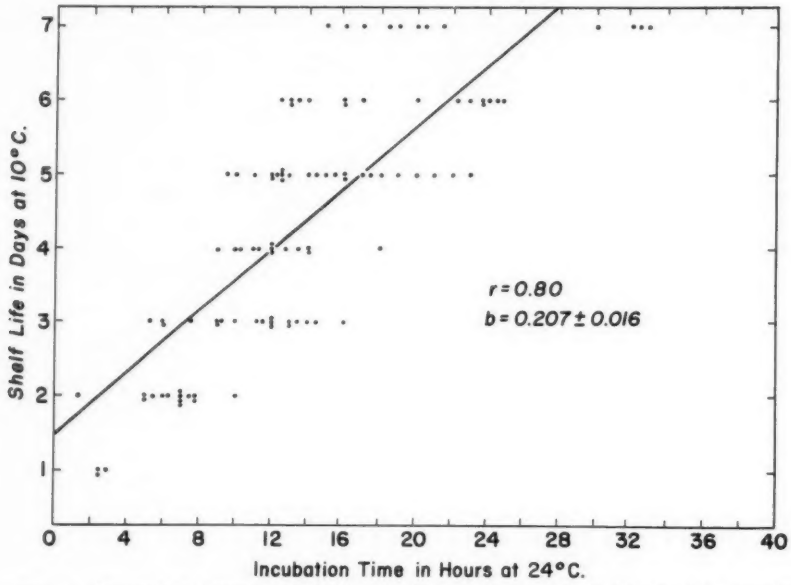


FIG. 1. Correlation between shelf life of Cottage cheese and incubation time required to reduce resazurin to pink.

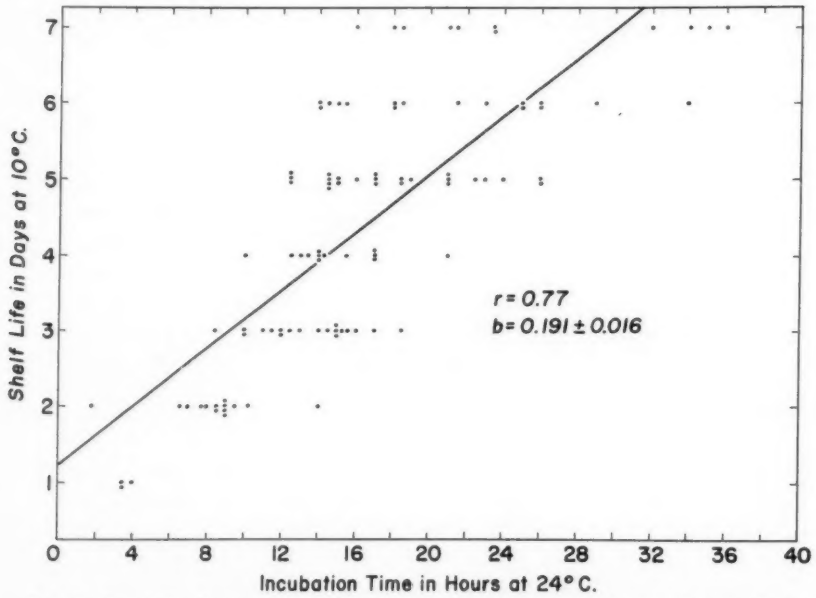


FIG. 2. Correlation between shelf life of Cottage cheese and incubation time required to reduce resazurin to white.

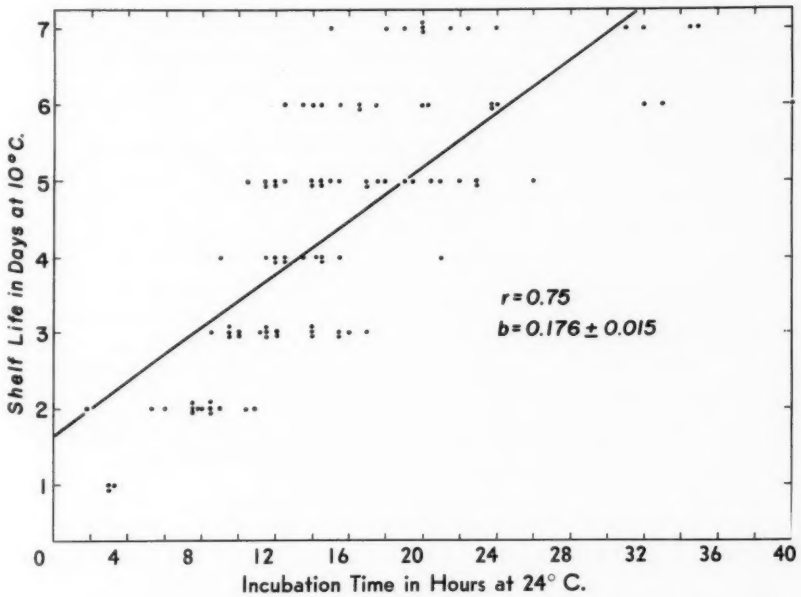


FIG. 3. Correlation between shelf life of Cottage cheese and incubation time required to reduce methylene blue.

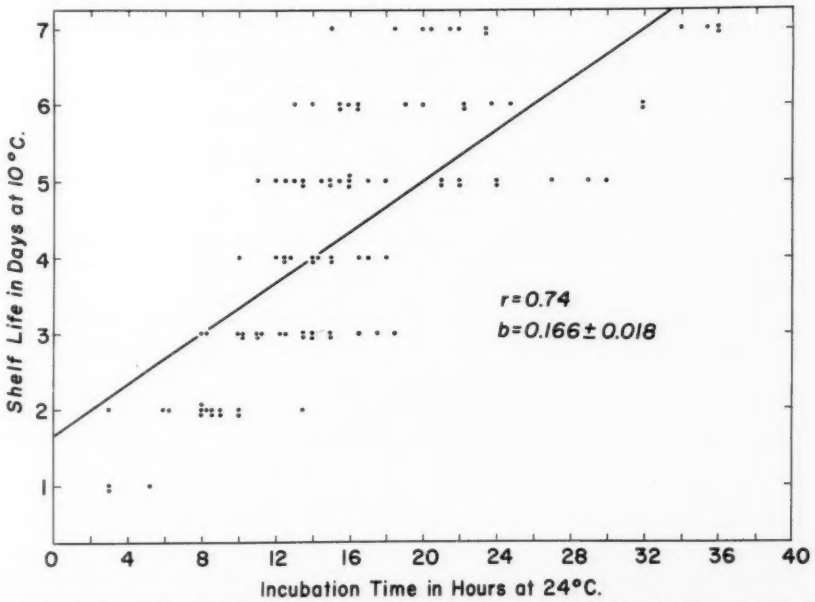


FIG. 4. Correlation between shelf life of Cottage cheese and incubation time required to reduce 2,3,5-triphenyl tetrazolium chloride.

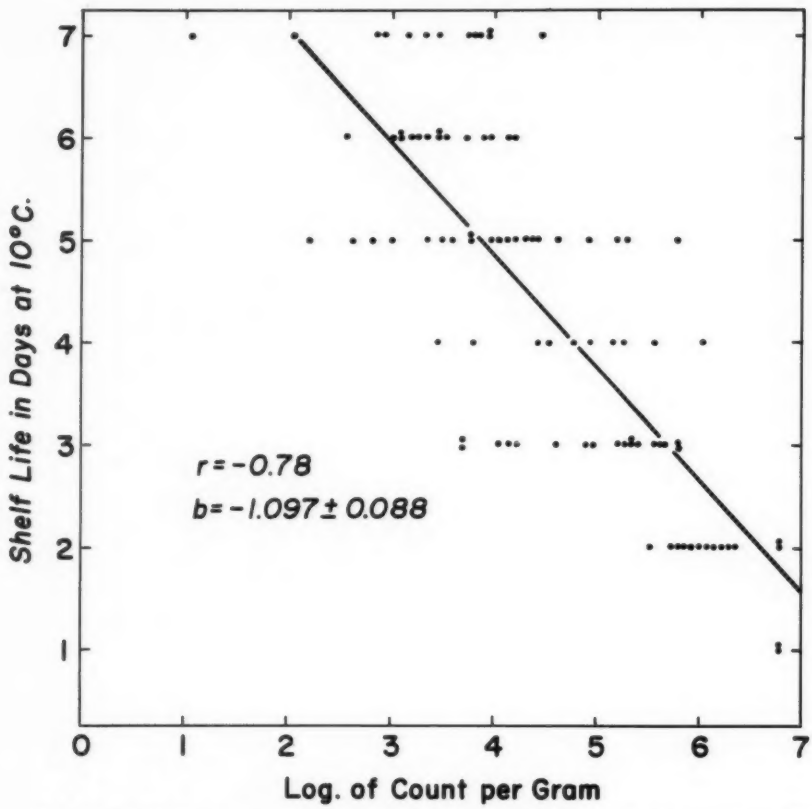


FIG. 5. Correlation between shelf life of Cottage cheese and log of total count on TGYE agar plates incubated three days at 22° C.

Although the range of time required to reduce resazurin to pink by the samples with a two-day shelf life is recorded as 1.3 to 10.0 hr. (Table 1), actually only one sample accomplished reduction in less than 5.0 hr. and only one sample required longer than 7.75 hr. Therefore, 18 of the 20 samples within this group accomplished reduction within the range of 5.0 to 7.75 hr.

It is noteworthy that all samples requiring more than 23 hr. to reduce resazurin to pink retained original organoleptic quality for six days or longer, indicating that incubation of the reduction tests overnight represents a method of identifying good-quality cheese.

Correlation between reduction time and shelf life. Data in Figures 1, 2, 3, and 4 show the correlation between incubation time in hours at 24° C. required to reduce the various dyes and shelf life of Cottage cheese in days at 10° C. The regression coefficients (b) indicate the fractions of a day in shelf life equivalent to each hour of reduction time. The values vary from 0.166 ± 0.018 with

the samples which reduced TTC, to 0.207 ± 0.016 with the samples which reduced resazurin to pink. The correlation values (r) vary from 0.80 with the samples in which resazurin was reduced to pink, to 0.74 with the samples which reduced TTC. The data indicate that the reduction of resazurin to pink is the preferable reduction test, because this method requires the least time and has the highest correlation ($r = 0.80$).

Correlation between shelf-life and total count on TGYE agar and psychrophile count on VRB agar. The data in Figure 5 show a correlation of -0.78 between shelf-life in days at 10° C. and total count on TGYE. The correlation between shelf life in days and psychrophile count on VRB is -0.67 (Figure 6). In both enumerations the plates were incubated at 22° C. for three days. The regression coefficients indicate that the shelf-life decreases approximately one day with a tenfold increase in total count (Figure 5) and approximately three-fourths of a day with a tenfold increase in psychrophile count (Figure 6).

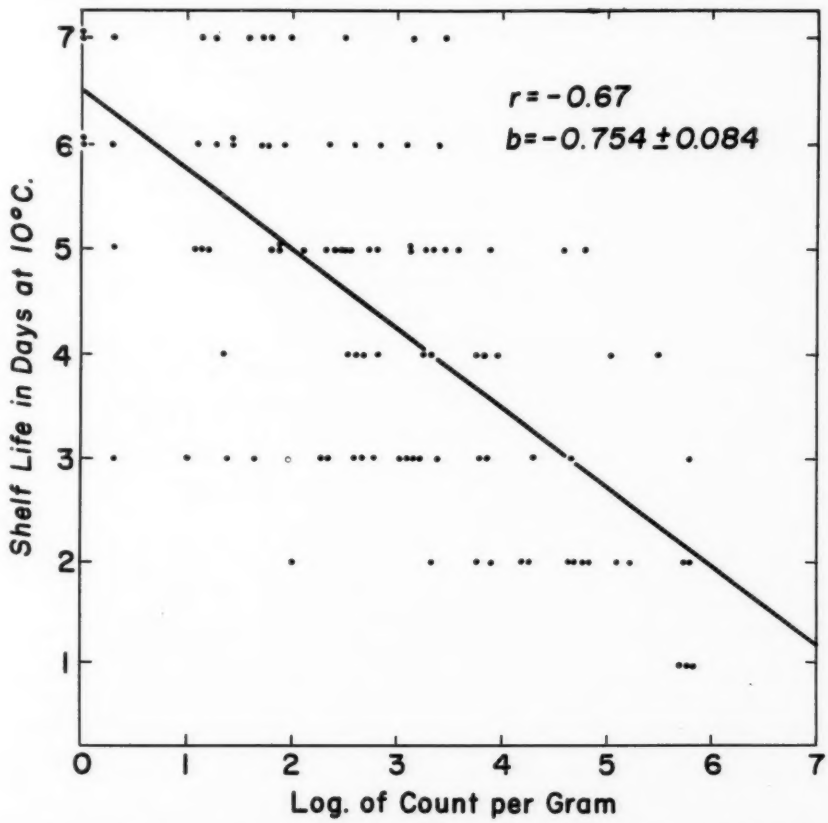


FIG. 6. Correlation between shelf life of Cottage cheese and log of psychrophile count on VRB agar plates incubated three days at 22° C.

Effect of preincubation on reduction time. A number of blended samples of cheese were held at 15 and 20° C. for 8 hr. prior to determining reduction time. Preincubation usually resulted in a decrease in reduction time, varying from one to several hours, but the decrease was not sufficient to justify the time devoted to preincubation. Frequently, there was a decline in pH attributable to lactic organisms and preincubation did not improve the correlation between reduction time and shelf life.

Effect of preliminary agitation of blended samples on reduction time. Twelve samples of Cottage Cheese were neutralized and blended as described previously. These samples were examined for total count, psychrophile count, and reduction time, before and after agitation on a mechanical shaker for 2.5 hr. at 24° C. The reduction times varied from 9.5 to 15 hr. with the nonagitated samples and from 3.5 to 10 hr. with the agitated samples. The average reduction times and the logarithmic averages of the bacteria counts are shown in Table 2. Agitation

TABLE 2

Effect of preliminary agitation for 2.5 hr. at 24° C. on plate counts and resazurin reduction time of neutralized, blended Cottage cheese

(Average of 12 samples)

Logarithmic averages of counts per gram of cheese (3 days at 22° C.)				Average reduction time (hours at 24° C.)	
Total count on TGYE		Psychrophile count on VRB			
Nonagitated	Agitated	Nonagitated	Agitated	Nonagitated	Agitated
4,500	20,000	34	76	11.9	7.6

resulted in a 344% increase in total count and a 124% increase in psychrophile count. The average reduction time was 4.3 hr. (50.7%) longer with the nonagitated samples than with the agitated. This represents an average saving of 1.8 hr., or 15% of the total time required for reduction of the nonagitated samples, and merits consideration, since the time element is a critical factor in completing the reduction test during the normal working day.

REFERENCES

(1) American Public Health Association. Standard Methods for the Examination of Dairy Products. 10th ed. New York. 1953.

(2) Broitman, Selwyn, Mallmann, W. L., and Trout, G. M. Simple Test for Detecting Keeping Quality for Milk. J. Milk and Food Technology, 21: 280. 1958.

(3) Greene, V. W., and Jamison, R. M. Influence of Bacterial Interaction on Reseazurin Reduction Time. J. Dairy Sci., 42: 1099. 1959.

(4) Hirshmann, D. J., and Lightbody, H. D. Resazurin Reduction Test and Microbiology of Egg Powders Prepared by Lyophilization. Food Research, 12: 372. 1947.

(5) Kereluk, Karl, and Gunderson, M. F. Resazurin Reduction Test as an Index of the Bacteriological Quality of Frozen Foods. J. Milk and Food Technol., 22: 299. 1959.

(6) LaGrange, W. S., and Nelson, F. E. Dye-Reducing Abilities of "Psychrophilic" Bacteria from Milk. Bacteriological Proc., p. 49. Annual Meeting Soc. American Bacteriologists, Philadelphia, Pa. May, 1960.

(7) MALLMANN, W. L., DAWSON, L. E., SULTZER, B. M., AND WRIGHT, H. S. Studies on Microbiological Methods for Predicting Shelf Life of Dressed Poultry. Food Technol., 12: 122. 1958.

(8) SCOTT, W. J., AND GILLESPIE, J. M. Tests for Quality in Egg Pulp. I. A Preliminary Note on the Application of the Reductase Test Using Resazurin as the Indicator. Australian Council Sci. Ind., 16: 15. 1943.

(9) SCOTT, W. J., AND GILLESPIE, J. M. Tests for Quality in Egg Pulp. II. Further Experiments on the Resazurin Reductase Test. Australian Council Sci. Ind., 17: 299. 1944.

(10) STRAKA, ROBERT P., AND STOKES, J. L. A Rapid Method for the Estimation of the Bacterial Content of Precooked Frozen Foods. Food Research, 22: 412. 1957.

(11) WALKER, H. W., COFFIN, W. J., AND AYRES, J. C. A Resazurin Reduction Test for the Determination of Microbiological Quality of Processed Poultry. Food Technol., 13: 578. 1959.

METHOD FOR DETERMINATION OF CITRIC AND LACTIC ACIDS IN DAIRY PRODUCTS[1]

D. R. FRAZEUR
Dairy Department, Purdue University, West Lafayette, Indiana

SUMMARY

A column chromatographic method for the determination of citric and lactic acids has been developed. The column was three-sectioned and was composed of unbuffered H_2SO_4 and silicic acid. The eluate was collected in three portions of varied size; lactic acid was the only hydroxy acid in the first eluate portion and citric acid was the only acid in the third portion. Citric acid was directly titrated and lactic acid was determined by the use of the $FeCl_3$-HCl-hydroxyorganic acid color reaction. Citric acid concentrations of 0.10 mg. to 0.4 g. per column were determined, with recoveries of 97 to 100%, with an average of 99.18 and a coefficient of variation of 0.47%. Lactic acid concentrations of 50 to 200 μg/5 ml. of eluate were analyzed with accuracies of 94.5 to 100.7%, with an average of 98.6 and a coefficient of variation of 1.2%. Advantages of this procedure are accuracy, low-cost apparatus, simplicity, and a minimum of sample preparation.

Recent interest in the amounts of citric and lactic acid in dairy products has been responsible for the development of a number of procedures for the separation and measurement of these acids. The individual importance of each acid makes quantitative determination desirable, but the fact that both are hydroxyorganic acids tends to make separation difficult. A number of workers (2, 10-14) have introduced variations of the Furth-Herrman reaction to measure the amount of citric acid. The Furth-Herrman reaction produces a yellow color when citric acid, pyridine, and acetic anhydride are combined. This reaction is strongly exothermic and somewhat difficult to control. The reagents are difficult to handle and the procedure must be very carefully controlled. Lactose, protein, and other materials interfere with the reaction. In another method (8), citric acid is oxidized to acetone dicarboxylic acid and brominated to pentabromoacetone, which is determined colorimetrically. The official method (1) uses this reaction and gives consistent results when carefully controlled. Conversion of citric acid to pentabromoacetone is laborious, time-consuming, and exacting.

Titratable acidity is frequently used to indicate the development of gross amounts of acid calculated as lactic acid. While this test is satisfactory for many manufacturing procedures, it is commonly recognized that titratable acidity is inadequate for the accurate determination of actual lactic acid in dairy products. Other methods (5, 16) have been developed which are based on the oxidation of lactic acid to acetaldehyde. The acetaldehyde is then determined by an iodometric titration in an excess of bisulphite. These methods are time-consuming and require that interfering substances be removed prior

Received for publication March 31, 1961.

[1] Contribution from the Purdue Agricultural Experiment Station. Journal Paper No. 1712.

to oxidation of lactic acid. The reddish-brown color developed when a hydroxy-organic acid is mixed with a $FeCl_3$-HCl solution, has been the basis for other lactic acid determinations (4, 6, 7, 9, 15). These methods are rapid and accurate, if interfering substances are not present. Among the substances interfering with the method are citric acid, lactose, and proteins.

Column chromatographic methods are relatively simple, use comparatively safe reagents, are relatively inexpensive, and give excellent results qualitatively and quantitatively. Bulen, Varner, and Burrell (3) have described a chromatographic column which gives excellent resolution and measurement of a number of organic acids. The method to be described is a modification of their method and it is suitable for the analysis of citric and lactic acids in dairy products.

METHOD

Apparatus:

(a) Glass chromatographic columns—Scientific Glass Apparatus Co. Cat. No. J. C.—2100 size No. 3, inside diameter 40 mm., inside length 250 mm., with perforated glass disc at the bottom.

(b) Burettes—25 ml., semimicro.

(c) Filter paper disc—40 mm. diameter, Whatman No. 1 or No. 4, cut with cork borer.

(d) Spectrophotometer with cuvettes.

(e) Centrifuge—to receive test tubes, preferably fitted with a 12-in.-diameter angle head.

(f) Mortar and pestle.

(g) Glass-stoppered test tubes, 25 by 200 mm.

Reagents:

(a) Chloroform—technical grade, distilled and washed four times with equal volumes of distilled water; filtered through triple layers of dry filter paper. Three 24-cm.-diameter circles of Whatman No. 1 paper were capable of filtering 1 gal. of chloroform. This paper could be dried for reuse.

(b) *n*-butanol—technical grade.

(c) Silicic acid—Mallenckrodt Chromatographic Special, washed in distilled water to separate the smaller-sized particles, and dried overnight at 105° C.

(d) Eluant—60% chloroform, 40% *n*-butanol by volume, saturated with a minimal amount of distilled water and filtered through triple layers of dry filter paper.

(e) 0.01 *N* alcoholic KOH.

(f) Absolute ethanol.

(g) Phenol red indicator—prepared by grinding 100 mg. of solid phenol red with 5.7 ml. of 0.05 *N* sodium hydroxide and making up to 100 ml. with distilled water.

(h) Ferric chloride indicator for lactic acid—prepared each day by mixing a drop of HCl and a drop of aqueous 50% $FeCl_3$ solution into 50 ml. of distilled water.

EXPERIMENTAL PROCEDURE

Preparation of sample. Liquid dairy products need no preparation, whereas ice cream requires melting before being sampled. Hard cheeses are ground twice through a fine-bladed food chopper and soft cheeses are prepared by blending in a Waring Blendor or similar instrument. Butter is melted in a separatory funnel and the aqueous and fatty layers are allowed to separate. The aqueous layer (butter serum) is used as the sample. Dried dairy products are reconstituted to normal total solids levels with distilled water.

Preparation of the column. The glass column is assembled and a filter paper disc is placed on the bottom. Three grams of washed and dried silicic acid and 2 ml. of distilled water are thoroughly ground together in a mortar. This material is transferred to a 250-ml. beaker, using a dry camel's-hair brush. A slurry is prepared by adding 75 ml. of chloroform. The silicic acid–chloroform slurry is quantitatively transferred to the glass column to form the first section. The silicic acid is allowed to settle to the bottom, leaving excess column building chloroform above. A filter paper disc is placed on the surface of the first section to separate the first and second sections. Silicic acid and added materials must always be a free-flowing powder when added to chloroform.

The second section of the column is formed in similar manner, except that 12 g. of silicic acid and 8 ml. of 0.7 *N* H_2SO_4 are ground together and slurried with 150 ml. of chloroform. The third or top section is constructed in essentially the same manner as the first two sections, but consists of 12 g. of silicic acid, 4 ml. of 0.7 *N* H_2SO_4, and 4 g. of sample, or sample and distilled water. Silicic acid and H_2SO_4 are ground together before the sample, or sample and water, are added. A filter paper disc is placed on the top of the column to prevent shifting of the upper section when eluant is added.

Separation and measurement of citric and lactic acids. The eluant is gently poured into the column when 0.25-0.50 in. of column building chloroform is still exposed above the silicic acid. A total of 600 ml. of eluate is collected in successive fractions of 75, 225, and 300 ml. To determine citric acid, the third fraction (300 ml.) is mixed with 10 ml. of absolute ethanol and ten drops of phenol red indicator and titrated with 0.01 *N* alcoholic KOH. A blank titration value, based on a corresponding eluate fraction from an identical column containing no sample, is subtracted from the titration reading and the remainder is calculated as citric acid. The blank value is constant and needs to be run only once for each lot of reagents.

For the determination of lactic acid, 5 ml. of the first eluate portion is transferred to a glass-stoppered test tube and 5 ml. of the $FeCl_3$-HCl color reagent is added. The test tube is stoppered, shaken vigorously for 2 min., and centrifuged at 1,500 r.p.m. to separate the aqueous and eluate layers. A suitable amount of the colored aqueous layer is put into a cuvette and read at 369 mμ.

using a spectrophotometer. Pure color reagent, treated as indicated above, was set at zero absorbance. No more than 10 min. should elapse between contact of color reagent with eluate and the completion of color measurement. Comparison of optical densities with a standard curve gives the concentration of lactic acid.

RESULTS AND DISCUSSION

Table 1 shows the position of several organic acids in the various eluate

TABLE 1

Location of organic acids relative to recommended chromatographic column

Not leaving the column	Present in eluate fraction		
	No. 1	No. 2	No. 3
Serine	Butyric	Glycollic	Citric
Threonine	Caprylic	Malic	
Tyrosine	Glutaric	Oxalic	
	Lactic	Tartaric	
	Oleic		
	Propionic		
	Pyruvic		
	Stearic		
	Succinic		

portions. Citric acid was the only acid in the third portion and could be directly titrated. Lactic acid and a sizable group of other acids were contained in the first eluate portion, whereas a smaller group of acids was present in the second eluate portion. In all, 18 acids were placed on the column and their elution studied. As might have been expected, the strongly acidic character of this column freed the acids from their salts so that results showed the sum of free acid and acid in salt form. The acids shown in Table 1 were selected to represent acids which might be present in a sample, plus those which might interfere, although their presence in dairy products might be questioned. Lactose and protein did not leave the column and triglycerides did not interfere with titration.

The accuracy of this procedure for the determination of citric acid was tested in three ways: (1) Aqueous solutions of known citric acid concentrations were made and analyzed. (2) Known amounts of citric acid were added to one-half of split samples of various dairy products and both halves were analyzed. (3) Carefully weighed amounts of crystalline citric acid were placed on completed columns and washed into the columns. Recoveries were then calculated by appropriate means. In all cases C. P. citric acid was used and columns were constructed and developed in the specified manner. Each of the three ways of proving accuracy were used five times, giving a total of 15 citric acid determinations. Recoveries ranged from 97 to 100% and averaged 99.18%, with a coefficient of variation of 0.4%. Concentrations of citric acid ranged between 0.10 mg. and 0.4 g. per column, although other concentrations might have been acceptable.

Table 2 shows the results obtained when samples of various dairy products

TABLE 2

Citric acid contents of various dairy products

Dairy products	Mg. citric acid/g of sample
Whole milk	1.81–2.42
Skimmilk	1.84–2.56
Cultured buttermilk	1.06–2.10
Chocolate milk	1.35–1.91
Ice cream (mix)	1.60–2.77
Cheddar cheese	0.19–0.63
Cottage cheese (creamed)	0.26–3.05
Nonfat dry milk (reconstituted)	1.14–2.01
Cottage cheese whey	1.12–1.90

were analyzed for citric acid. At least ten samples of each product were tested and, where possible, selection was made to obtain a range of citric acid concentrations.

The determination of lactic acid by this procedure is based on the principle that no other hydroxy acid is found in the first eluate portion and that no interfering substances, such as lactose and proteins, are present. Various hydroxy amino acids gave color with $FeCl_3$ and HCl but, as indicated in Table 1, these acids do not leave the column. Color formation followed Beers Law, when 50 to 200 μg. of lactic acid, 5 ml. of eluate, and 5 ml. of color reagent were combined. Commercial 85% lactic acid was too impure for use in checking recoveries; therefore, C. P. zinc lactate was used in all trials. This procedure determined both lactic acid and lactate as free lactic acid, so no measurement of neutralization could be made. A table of the lactic acid contents of various dairy products was not prepared, since lactic acid contents appeared to vary with the history of the sample rather than as a typical value for a type of product. Analyses of ten samples of fresh milk yielded results too low (minimum 0.019%) to be quantitatively determined by this method. In 15 trials, lactic acid recoveries ranged from 94.5 to 100.7% and averaged 98.6%, with a coefficient of variation of 1.2%.

The cost of this procedure was lowered by recovering a major portion of the used chloroform. The eluate and column building chloroform were combined and neutralized, so that no acid would be distilled over into the reclaimed chloroform. An all-glass distillation apparatus, including a scrubbing column, was used and only that chloroform coming over below 70° C. was saved. Attempts to use recovered butanol were unsuccessful, possibly due to the difficulty of sufficiently separating ethanol and *n*-butanol. Contamination of *n*-butanol by ethanol would markedly change the polarity of the eluant and erratic results might be expected.

REFERENCES

(1) Association of Official Agricultural Chemists. Official Methods of Analysis. 8th ed. Association of Official Agricultural Chemists, Washington, D. C. 1955.

(2) Babad, J., and Shtrikman, N. The Estimation of Citric Acid in Dairy Products. J. Dairy Research, 18: 72. 1951.

(3) Bulen, W. A., Varner, J. E., and Burrell, R. C. Separation of Organic Acids from Plant Tissues. Anal. Chem., 24: 187. 1952.

(4) Cope, B., and Choi, R. P. Adaption of the Ling Method for the Determination of Lactic Acid in Non-Fat Dry Milk. American Dry Milk Institute. Unpublished data. 1957.

(5) Friedman, T. E., Catonio, M., and Shaffer, P. A. The Determination of Lactic Acid. J. Biol. Chem., 73: 335. 1927.

(6) Harper, W. J., and Randolph, H. E. Lactic Acid in Cottage Cheese. Am. Milk Rev., 22: 43. 1960.

(7) Hillig, F. The Colorimetric Determination of Lactic Acids in Milk and Milk Products. J. Assoc. Offic. Agr. Chemists, 20: 130. 1937.

(8) Lampitt, L. H., and Rooke, H. S. The Citric Acid in Milk and Its Determination. Analyst, 61: 654. 1936.

(9) Ling, E. R. The Determination of Lactic Acid in Milk. J. Sci. Food and Agr., 2: 279. 1951.

(10) Marier, J. R., and Boulet, M. Direct Determination of Citric Acid in Milk with an Improved Pyridine–Acetic Anhydride Method. J. Dairy Sci., 41: 1683. 1958.

(11) Marier, J. R., and Boulet, M. Analysis for Citric Acid in Presence of Casein. J. Dairy Sci., 43: 1414. 1960.

(12) Murthy, G. K., and Whitney, R. McL. Colorimetric Determination of Citrate in Milk by Babad and Shtrikman. J. Dairy Sci., 40: 1495. 1957.

(13) Reinart, A., and Nesbitt, J. M. Modification of the Method of Caffran and Denstedt for Citrate in Milk. J. Dairy Sci., 40: 1645. 1957.

(14) Saffran, M., and Denstedt, O. F. A Rapid Method for the Determination of Citric Acid. J. Biol. Chem., 175: 849. 1948.

(15) Steinsholt, K., and Calbert, H. E. A Rapid Colorimetric Method for the Determination of Lactic Acid in Milk and Milk Products. Milchwissenshaft, 15: 7. 1960.

(16) Troy, H. E., and Sharp, P. F. The Quantitative Determination of Lactic Acid in Dairy Products. Cornell Agr. Expt. Sta. Memoir 178. 1935.

FACTORS RELATED TO THE FLAVOR STABILITY OF FOAM-DRIED WHOLE MILK. I. EFFECT OF OXYGEN LEVEL[1]

A. TAMSMA, M. J. PALLANSCH, T. J. MUCHA, AND W. I. PATTERSON
Dairy Products Laboratory, Eastern Utilization Research and Development Division, USDA
Washington, D. C.

SUMMARY

After developing methods capable of packaging milk powders in atmospheres of nitrogen containing less than 0.1% oxygen, a study was made of the effect of the in-package oxygen levels on the storage life of foam-dried whole milk. It was found that the shelf life of this product could be extended by reducing the oxygen levels in the packages below those now generally considered acceptable for the storage of spray-dried whole milk powders. During an 8-mo. period, 14 foam-dried milks were made from fluid milks, each of which received a minimum heat treatment of 145° F. for 30 min. The dry milks contained 2-3% moisture, were packaged in an atmosphere that contained about 0.1, 1, and 21% oxygen, and stored at 80° F.; they were flavor-scored each 2 mo. for 6 mo. by a ten-judge expert panel, using a slight modification of the American Dairy Science Association scoring guide. A statistically, highly significant improvement in storage stability was noted in milk powders packed in 0.1% oxygen compared with those packed in 1% oxygen and in air. Numerical trends in oxidized and other off-flavors are reported.

A new method for drying whole milk has been devised recently in the laboratories of this Division (11). This foam-dried powder can be easily reconstituted to a milk of beverage quality. The powder as initially prepared by a batch process is bulky and subject to off-flavor development during storage. Research was undertaken to overcome these defects. This paper reports results obtained during a study of the effect of in-package oxygen levels on the storage life of foam-dried whole milk powders.

The effect of in-package oxygen levels on the shelf life of spray-dried whole milk has been the subject of numerous investigations. Although there has been a general consensus of opinion among investigators that reduced levels of oxygen in the package retard the development of oxidized flavor in whole milk powders, no agreement exists as to the minimum levels needed during storage. Lea *et al.* (9) concluded that the development of oxidized flavors in spray-dried whole milk could be controlled by reducing the oxygen content in the free space of the container to 1 to 3%. Coulter (4) and Coulter *et al.* (5) concluded that less than 1% oxygen in the packing gas was necessary to prevent oxidation of the whole milk powder. Schaffer (10) reported 3% as the upper limit of in-package oxygen content for extended storage life at room temperature. In the light of this information, and in view of the fact that nothing was known about stabilizing the flavor of foam-dried milk, methods for packaging products in nitrogen containing controlled amounts of oxygen were developed and a study

Received for publication October 16, 1960.

[1] A preliminary report was presented at the 54th Annual A.D.S.A. Meeting at the University of Illinois, Urbana, June, 1959.

of the effect of in-package oxygen levels on the storage life of foam-dried whole milk was undertaken.

METHODS

Preparation of foam-dried whole milk. The original procedure (13) for the production of foam-dried milk was employed with the following exceptions: (a) The concentrate was heated to 145° F. and homogenized, at 4,000 and 500 p.s.i. pressure on the first and second stages, respectively; (b) nitrogen was metered into the concentrate through a capillary tube and dispersed by passing the 110° F. mixture twice through a two-stage homogenizer, using 500 p.s.i. pressure on both stages. After drying, the moisture content of the comminuted foams varied between 2.0 and 3.2% before packaging. Moisture, dispersibility, and free fat were determined as described previously (13). Bulk density was determined by tamping 10 g. of dry milk in a 100-ml. graduate cylinder to the minimum volume and dividing ten by the minimum volume in milliliters.

Packaging. Dry milks were packed within one day after preparation in 208 by 208 tin cans, ⅔ of the cans (Series 1 and 2) were sealed with lids in which 1/32-in. holes drilled into a patch of solder were left open, and the rest of the cans (Series 3) were covered with lids but not sealed. The samples were placed in a vacuum shelf drier, Series 1 and 2 on circular discs which could be rotated, permitting every can to appear in front of a window containing two ⅝-in. holes closed with rubber stoppers. After holding the cans overnight below 1-mm. pressure at room temperature, the vacuum was broken with nitrogen (Seaford grade—oxygen content below 0.1%) to a slight excess over atmospheric pressure. The stoppers in the window were removed and a soldering iron and a rod for turning the disc were inserted. Series 1 was sealed while excess pressure was maintained continually in the drier. Series 2 was prepared by again drawing a vacuum to below 1-mm. pressure, breaking with nitrogen containing 1% oxygen, and sealing while using the same precautions. The vacuum was drawn once more, broken with air, and Series 3 sealed with a can sealer. The cans were tested for leaks by placing them under deaerated water in a desiccator and pulling a vacuum. Samples were discarded if bubbles escaped from seams or seals. Packing under three levels of oxygen: below 0.1%, at 1%, and in air for all batches of dry milk was accomplished by this procedure. The oxygen content of the interstitial gas in the containers was determined by the Haldane method (7) in two samples of the low oxygen packs, drawn from duplicate cans without oxygen contamination. Using the packing procedure described, it was found that the lowest level of oxygen packs always contained below 0.1% oxygen. The intermediate oxygen level packs ranged from 1.1 to 1.3% oxygen. The moisture content of the dry milk in the cans was reduced to approximately 2% because of the overnight evacuation. After packaging, the cans were stored at 80° F. After initial tasting, the packaged, stored dry milks were periodically tasted and scored at ages up to six months, by ten selected judges.

TASTE PANEL PROCEDURES

Selection of judges. Prior to this study, flavor thresholds of potential judges were evaluated for specific flavors, so that a statistically homogeneous taste

panel could be developed. Since cooked and oxidized flavors were expected to predominate in this and projected studies, they were the ones utilized in screening the judges. Various intensities of the off-flavors in milk were prepared by dilution. This diluting technique was successfully used by Kirkpatrick (8) and others (1-3, 6). The samples of the various concentrations thus prepared were presented to the judges in randomized pairs. The judges were asked to determine which sample of each pair possessed more off-flavor. The judges choosing the greatest number of samples correctly were used for the panel. These judges were later proven to be homogeneous, since they had similar judgment of a particular sample. This group of judges, which was used throughout the entire flavor study, should be considered qualified, because the likes and dislikes of flavor were not important as they would be for a consumer panel.

Testing experimental milks. After the group selection was completed, ten judges met almost daily in a temperature-controlled taste-panel room (70 ± 2° F.) where they tasted and scored about ten samples at each sitting.

In each experiment, the dry milks were first tasted within one or two days after preparation (initial) and at intervals after storage (usually after 2, 4, and 6 mo.). They were reconstituted (12.6% T.S.) with the proper proportion of chlorine-free spring water during the afternoon preceding the tasting period, which took place at 11 o'clock on the following morning. Samples were kept in a refrigerator overnight and warmed to 95° F. the next day. The milk cooled to 75-80° F. in the cups before tasting. The judges independently evaluated the flavor of the samples and recorded a numerical score corresponding to the flavors they found in each sample. The scoring guide used in these studies was similar to the one adopted by the American Dairy Science Association and is shown in Table 1.

Evaluation of the scores. The initial score, or the score after 2, 4, or 6 mo. of storage at 80° F. for a particular sample, was represented by the average

TABLE 1

Flavor score guide used throughout these studies

Criticisms	Slight	Definite	Pronounced
Acid	36-38	33-35	29-32
Astringent	37-39	33-36	28-32
Bitter	34-37	29-33	25-28
Chalky	37-39	33-36	29-32
Cooked	38-40	35-37	32-34
Feed	38-40	35-37	32-34
Flat	38-40	36-37	34-35
Foreign, objectionable	20-25	10-19	0-9
Foreign, unobjectionable	36-38	33-35	30-32
Metallic	32-34	28-31	24-27
Oily (greasy)	34-35	32-33	30-31
Oxidized	33-36	29-32	24-28
Rancid (lipolysis)	20-25	10-19	0-9
Salty	36-38	33-35	30-32
Scorched	34-36	31-33	28-30
Stale	34-36	31-33	28-30
Unclean	34-36	31-33	28-30
Weedy	32-34	28-31	24-27

score of the ten judges. The flavor data obtained by the ten taste-panel judges were analyzed for significance by the F-test or t-test (12).

RESULTS AND DISCUSSION

The effect of oxygen level in the package on dry milk flavor. The effects of the three levels of oxygen on the flavor score average during 6 mo. at 80° F. are presented in Figure 1. Fourteen dry milks were prepared by a standard procedure for eight months. Storage stability increased with decrease in oxygen content, which indicates that oxidative processes are causes for deterioration of foam-dried whole milk during storage. The initial average score, 35.6, decreased to 35 in about 1 wk. when the milks were kept in air, in about 3 wk. if kept at 1% oxygen, and after 4½ mo. at 0.1% oxygen. A great improvement in storage stability resulted from packing at 0.1% as compared to 1% oxygen, the level obtained with commercial nitrogen pack. Results from the literature (4, 5, 9, 10) indicate that a higher level of oxygen can be tolerated in spray-dried whole milk packages, which may be due to the fact that it is less bulky and consequently each can has less gas available for the fat. Also, spray-dried milk may have received more heat treatment. The effect of heat treatment on the storage stability of foam-dried whole milk will be the subject of a subsequent paper. The initial scores of the powders varied between 35 and 37, with one exception. No relationship was apparent between scores and bulk density, free fat, dispersibility, or moisture.

Data from the flavor tests, as evaluated by statistical treatment, for the effect of oxygen level in the package are summarized in Table 2. The results of the t-test showed that the samples with 1% oxygen were significantly better than those packaged in air; likewise, samples packed in 0.1% oxygen scored higher than those packaged with 1% oxygen. Statistical analysis of the data permits prediction of the range in flavor scores which can be expected with different oxygen contents in the canned samples after storage.

TABLE 2

A comparison of the flavor scores of foam-dried milks containing various levels of oxygen after storage for 6 mo. at 80° F.

Oxygen levels compared	Standard error of average flavor score difference	t-Value required for significance at 0.1% level	Degrees of freedom	Probability level at which significance occurred	t-Value calculated	Confidence limits for average flavor score difference**
			(n − 1)	*(%)*		*(95% confidence level)*
0.1 vs. 1	0.214	4.22	13	0.1	4.86	1.04 ± 0.43
1 vs. air	0.438	4.22	13	0.1	4.78	2.09 ± 0.88
0.1 vs. initial	0.210	4.22	12*	1.0	3.94	0.83 ± 0.42
1 vs. initial	0.270	4.22	12*	0.1	6.63	1.70 ± 0.54
Air vs. initial	0.288	4.22	12*	0.1	13.33	3.84 ± 0.57

* Data on one was excluded because its initial score was exceptionally low.

** The confidence limits indicate the range within which the true difference in flavor scores for the compared oxygen levels would be expected to fall: The average plus and minus two times the standard error.

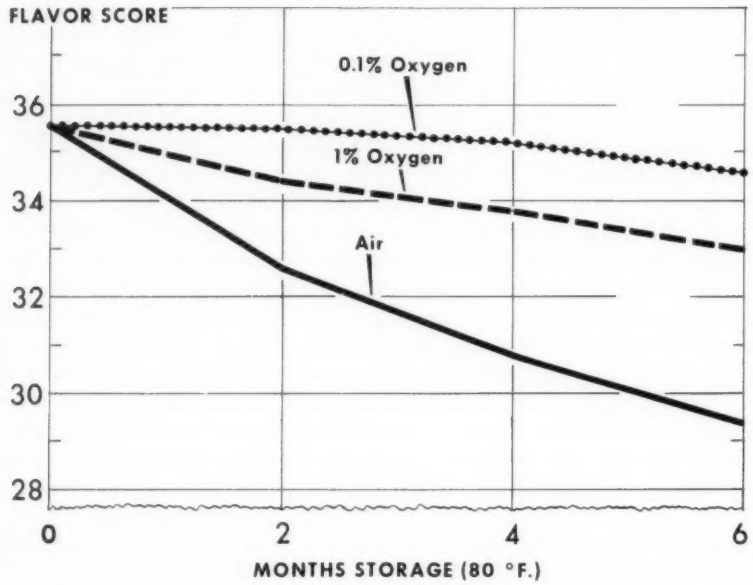


FIG. 1. Flavor score of foam-dried whole milk during storage. Averages of 14 samples.

A comparison of the flavor score means of dry milks packed at various levels of oxygen, initially and after 6 mo. storage at 80° F., is shown in Table 2. This indicates the decrease of the flavor score from its initial value that would be expected after 6 mo. storage. Data on one dry milk with an exceptionally low initial score was not included. This indicates that the samples packaged at the 0.1% oxygen level and stored for 6 mo. at 80° F. did not differ significantly from their initial values. There was a greater decrease in flavor score in the 1% oxygen packed samples similarly stored for the same period. The air packed samples were the poorest and they had the greatest decrease in flavor score. These differences were all statistically analyzed at the 0.1% level of significance. A score range difference at 0.4 to 1.2; 1.3 to 2.3; and 3.3 to 4.4 was found between initial and 6-mo.-old samples held at 80° F. and that were packaged in 0.1, 1, and 21% oxygen (air) atmospheres, respectively.

Table 3 is a compilation of the most frequent off-flavors reported by the judges when tasting milks reconstituted after storage at 80° F. in atmospheres of 0.1% and 1% oxygen, and in air. The oxidized flavor was predominant at all levels of oxygen. Trends in this and other flavors during 6 mo. of storage are noteworthy.

ACKNOWLEDGMENT

The authors acknowledge the assistance of C. Allen, J. Vestal, and H. E. Vettel in preparing the samples; J. Davoli and R. Trelogan for analyses; A. Lindquist for arranging the

taste panels; and H. Anderson, R. Bell, E. Boyd, L. Burgwald, E. Corbin, S. Detwiler, L. Edmondson, F. Fenton, K. Fox, A. Freeman, M. Graham, H. Haller, F. Hanrahan, R. Hargrove, S. Hoover, C. Hufnagel, M. Keeney, A. Leviton, L. Rogers, D. Schwartz, R. Tittsler, H. Weihe, and H. Welton as judges on the taste panels.

TABLE 3

A distribution of frequencies of various predominating off-flavors as influenced by oxygen level and period of storage at 80° F.

Oxygen level	Storage 80° F.	Flavor criticism (per cent occurrence)						
		Astringent	Cooked	Feed	Oxidized	Stale	Unclean	Others
(%)	*(mo.)*							
Initial	0	13[a]	27[a]	3[a]	21[a]	13[a]	5[a]	18[a]
0.1	2	14	24	2	36	6	7	11
	4	12	19	2	35	12	7	13
	6	7	13	1	46	11	7	15
1	2	10	14	1	56	3	8	8
	4	8	9	1	52	11	4	15
	6	5	7	1	64	12	3	8
Air	2	8	12	2	60	7	3	8
	4	4	5	1	70	9	3	8
	6	2	7	1	75	8	2	5

[a] Only one sample was tested initially for all oxygen levels.

REFERENCES

(1) Bohren, R. B., and Jordan, R. A. Technique for Detecting Flavor Changes in Stored Dried Eggs. Food Research, 18: 583. 1953.

(2) Boggs, M. M., and Hanson, H. L. Analysis of Foods by Sensory Difference Tests. Advances in Food Research, 2: 219. 1950.

(3) Carlen, A. F., Kempthorne, O., and Gordon, J. Some Aspects of Numerical Scoring in Subjective Evaluation of Foods. Food Research, 21: 273. 1956.

(4) Coulter, S. T. The Keeping Quality of Dry Whole Milk Spray Dried in an Atmosphere of an Inert Gas. J. Dairy Sci., 30: 995. 1947.

(5) Coulter, S. T., Jenness, R., and Crowe, L. K. Some Changes in Dry Whole Milk During Storage. J. Dairy Sci., 31: 986. 1948.

(6) Dawson, E. H., and Harris, B. L. Sensory Methods for Measuring Differences in Food Quality. USDA Information Bull. 34: 134. 1951.

(7) Haldane, J. S., and Graham, J. I. Methods of Air Analysis. 4th ed., pp. 8-53. Charles Griffin and Co., London. 1935.

(8) Kirkpatrick, Mary E., Lamb, Jessie C., Dawson, Elsie H., and Eisen, J. N. Selection of a Taste Panel for Evaluating the Quality of Processed Milk. Food Technol., 11: 3. 1957.

(9) Lea, C. H., Moran, T., and Smith, J. A. B. The Gas-Packing and Storage of Milk Powder. J. Dairy Research, 13: 162. 1943.

(10) Schaffer, P. S. Abstract of Papers Presented by G. E. Holm. Tech. Conf. of the Dry Whole and Ice Cream Ind. 3, QMC Subsistence and Research Lab., Chicago. 1945.

(11) Sinnamon, H. I., Aceto, N. C., Eskew, R. K., and Schoppet, E. F. Dry Whole Milk. I. A New Physical Form. J. Dairy Sci., 40: 1036. 1957.

(12) Snedecor, G. W. Statistical Methods. 4th ed. Iowa State College Press, Ames, Iowa. 1946.

(13) Tamsma, A., Edmondson, L. F., and Vettel, H. E. Free Fat in Foam-Dried Whole Milk. J. Dairy Sci., 42: 240. 1959.

CHEMICAL STATE AND NUTRITIONAL AVAILABILITY OF IRON IN A PREPARED INFANT FORMULA

O. W. VAUGHAN AND R. E. KNAUFF
Ross Laboratories, Columbus, Ohio

SUMMARY

The distribution and chemical state of ferrous iron added to a prepared infant formula was studied, using Fe^{59} as a tracer. The iron was found to be almost entirely associated with the milk proteins. Before protein hydrolysis, only 3% of the iron was dialyzable against either distilled water or a citrate solution, but after hydrolysis in vitro, 20% of the iron was dialyzable against water and 79% against citrate solution. Digestion in vivo increased the percentage of dialyzable iron still further. Most of the iron added to milk products exists as a soluble protein iron complex and it is available nutritionally after the protein portion of the complex is digested.

The percentage of iron readily available as ferrous iron is considered to be important in assessing the dietary value of an iron-containing food. For this reason, most tables of food values show the amount of iron which reacts with α-α'-dipyridyl along with the amount of total iron. The values obtained from this reaction, however, may not accurately represent the nutritionally available iron in a given food, since the effect of intestinal enzymatic digestion is not considered. There is abundant clinical evidence showing that iron added to milk and milk products is nutritionally available (6, 8-10), even though nearly all of this added iron exists in a tightly bound conjugate.

The distribution and chemical state of iron added to milk or milk fractions have been examined by a number of workers. Allan (1), while studying the kinetics of ascorbic acid oxidation in milk and butter serum, concluded that both added and natural iron were nonionic and were associated with the fat globule membrane. Van der Waarden (12) studied the distribution of natural and added copper and iron in butter and found that the proteins contained all of the iron. More recently, King *et al.* (7), using Fe^{59} as tracer, found that iron added to milk was essentially all associated with the skimmilk and only slightly dialyzable.

In the present investigation, a radioactive tracer technique was used to determine the distribution of added iron in a prepared infant formula.[1] The dialyzable iron was determined both before and after enzymatic digestion of the milk proteins in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Preparation of the Fe^{59}-labeled milk product. Ferrous sulfate, $Fe^{59}Cl_2$, and citrate were added to Product S before final heat processing. The resulting

Received for publication April 8, 1961.

[1] Similac with Iron, a milk product simulating human milk in composition, containing added iron (Product S).

solution was thoroughly mixed, canned, and sterilized in pilot plant equipment. Labeled Product S contained 0.2% citrate, 25 mg. of ferrous iron per liter, and 42 μc. of Fe^{59} per liter.

Radioactivity measurements. Samples were pipetted into stainless steel planchets, evaporated to dryness by a stream of warm air, weighed, and counted with a Nuclear-Chicago D-47 Gas Flow Counter and Model 186 Scaler. Radioactivity is expressed as counts per minute, corrected for decay and background.

Fractionation. Ten milliliters of Fe^{59}-labeled Product S were mixed with 70 ml. of ethanol. After 2 hr., the mixture was centrifuged. The precipitate was washed with 60% ethanol until essentially no material was present upon evaporation of the wash solutions. The protein precipitate was then washed thrice with 70-ml. portions of acetone and six times with a 70:30 mixture of ether and absolute ethanol. Most of the alcohol used to precipitate the protein was evaporated from the supernatant, and the resulting aqueous solution was extracted with ether until no further lipid material could be removed. All the organic solvents used for washing the protein precipitate and extracting the supernatant were combined and the solvents evaporated. Aliquots of the lipid fraction, the alcoholic supernatant, and the protein fraction were counted for Fe^{59} activity.

Dialysis. Five-milliliter portions of enzyme-digested or undigested Fe^{59}-labeled Product S in dialyzing tubing of ½-in. diameter were dialyzed against 2 liters of distilled water or 0.2% citrate solution for at least 24 hr. at room temperature. In other experiments, 5-ml. portions of a 10% solution of nondialyzable dextrins were dialyzed against 200 ml. of labeled Product S. The dextrin solution had an osmotic pressure of the same order of magnitude as the milk product; therefore, litle water was lost from the bag during dialysis. Under similar conditions, essentially all the water is lost from a dialysis bag of distilled water.

Enzymatic digestion of milk protein in vitro. The protein in 5-ml. samples of Fe^{59}-labeled Product S was hydrolyzed by a 2% solution of pepsin and a 2% solution of trypsin according to published procedures (5). The enzyme-treated solution was dialyzed against distilled water or citrate solution, and the radioactivity of the dialysates determined.

Digestion in vivo. Adult male rats of Holtzman origin were fasted 24 hr. and fed 3-ml. portions of Fe^{59}-labeled Product S by gavage. At 1, 2, 4, and 6 hr., groups of three rats were killed and their gastrointestinal tracts ligated at the pyloric sphincter and at the ileocecal junction. The gastrointestinal tract was then removed and stretched out straight on a table top. The regions containing radioactivity were located with a portable rate meter. The contents of the radioactive segment of the small intestine from each rat were washed into a 50-ml. beaker with water. The volume in each beaker was adjusted to 20 ml. and an aliquot was counted for Fe^{59} activity. Ten milliliters of the aqueous solution were dialyzed against two liters of distilled water, and the radioactivity remaining in the dialysis bag was measured.

RESULTS AND DISCUSSION

Distribution of added iron. The distribution of added iron in various fractions of milk Product S is shown in Table 1. The high level of Fe^{59} activity in the protein fraction shows that most of the added iron is either bound by the milk proteins or by some other substance precipitated by alcohol.

TABLE 1

Distribution of added Fe^{59} in Milk Product S

Sample description	Activity (*c.p.m.*)[a]	Per cent in fraction
Product S, unfractionated 25 mg. Fe/liters, 42 μc. Fe^{59}/liters	93,000	100.00
Ethanol supernatant	1,000	1.07
Lipid fraction	50	0.00
Protein fraction	92,000	98.90

[a] Counts per minute, corrected for decay and background, in a 10-ml. sample of Product S or in a fraction separated from 10 ml. of Product S.

King *et al.* (7) added Fe^{59}-labeled ferric chloride (0.2-1.0 p.p.m.) to milk, then fractionated the milk by two different procedures. In one procedure casein was removed from skimmilk by isoelectric precipitation and in a second procedure by supercentrifugation. Whey proteins were precipitated by saturation with ammonium sulfate and removed by filtration. Almost all of the added iron was found in the protein fractions.

It seems that iron added to milk or milk products is present in the mixture primarily as a protein-iron conjugate, since the iron is recovered along with the proteins no matter which procedure is employed for fractionation.

Dialyzability of added iron. Samples of Fe^{59}-labeled Product S were dialyzed against two liters of distilled water for various periods of time ranging from 0 to 72 hr. The water was changed every 12 hr. After 24 hr., 3% of the Fe^{59} had been removed by dialysis. The increase in the amount of iron removed by longer periods of dialysis was too small to be detected. When labeled Product S was dialyzed against a 0.2% citrate solution, results were no different from those obtained using distilled water.

To arrive at a more precise quantitative measurement of the dialyzable iron, a dialysis bag of aqueous dextrin solution was dialyzed against 200 ml. of Product S. After 24 hr., the concentration of Fe^{59} in the dextrin solution was only 3% as great as the concentration in undialyzed Product S.

It may be concluded from the dialysis experiments that most of the added iron is present as a conjugate that is not dialyzable. The iron conjugate is not a precipitate of ferrous phosphate, since this water-insoluble salt is soluble in 0.2% citrate.

Iron liberated by the hydrolysis of protein in vitro. To determine whether iron is released by the hydrolysis of milk protein, samples were treated with the enzymes pepsin and trypsin, and the hydrolysate mixture dialyzed against distilled water or citrate solution. It can be seen from Table 2 that only 3% of the iron added to Product S was dialyzable before enzymatic treatment, but

TABLE 2

Influence of protein hydrolysis on the amount of dialyzable iron in Milk Product S

Sample description	Mass/ml (g.)[a]	Activity/ml (c.p.m.)[b]	Dialyzable Fe^{59} (%)	Dialyzing solution
Undialyzed Product S	0.048	7,500	....	.....
Enzyme-treated	0.013	6,000	20	Distilled water
Enzyme-treated	0.0120	1,600	79	Citrate solution
Not enzyme-treated	0.0210	7,250	3	Distilled water
Not enzyme-treated	0.0185	7,230	3	Citrate solution

[a] Grams of nondialyzable material per milliliter.
[b] Counts per minute corrected for mass, background, and decay.

that 20% of the iron passed out of the dialysis bag after protein hydrolysis. The presence of citrate in the distilled water against which the samples were dialyzed was without effect on the percentage of iron dialyzed from controls, but the dialyzable iron in enzyme-treated samples increased from 20% in distilled water to 79% in the citrate solution.

The hydrolysis of casein releases a considerable quantity of phosphate available to react with ferrous iron to form the very insoluble salt, ferrous phosphate. Ferrous phosphate is quite soluble in aqueous citrate solution and, therefore, would pass out of a dialysis bag submerged in such a solution. This provides a plausible explanation for the increased percentage of dialyzable iron in enzyme-treated samples in the presence of citrate.

Iron liberated by digestion in vivo. It is apparent that the iron added to milk Product S is tightly bound by the proteins, since only a small percentage of the iron is dialyzable and the percentage is not increased by the presence of citrate. After enzymatic hydrolysis of the milk proteins, however, most of the iron is released from its conjugate, with 79% of the iron dialyzable in the presence of citrate.

To determine whether iron in Product S remains in a soluble form after digestion in vivo, and the percentage of dialyzable iron in the digest, the experiment in vivo already described was carried out. There were large variations among the samples from different experimental animals in values for percentage of dialyzable iron, and these values did not seem to depend on the length of the digestion period. Dialyzable iron in the samples ranged from 20.5 to 37% of the total in distilled water and from 75 to 90% in citrate solution.

When the rats were killed to obtain samples digested in vivo, it was found that some of the dose still remained in the stomach, even after 6 hr. Therefore, the duodenal segments of the gastrointestinal tract from these rats always contained protein only partially digested. Nevertheless, the percentage of dialyzable iron in the samples digested in vivo was higher than in samples digested in vitro. This difference in dialyzable iron can be explained by assuming that phosphate ions are absorbed by the intestine as they are released by hydrolysis of the casein, or that citrate, peptides, or some other substance which renders ferrous phosphate soluble is present in the gut lumen after protein digestion.

The data suggest that most of the iron added to milk products is available nutritionally. The quantity of iron dialyzable against a very dilute citrate solution after protein digestion seems to be comparable to the quantity of iron which may be absorbed by the intestine from a milk product; Gorten *et al.* (3), in a study with premature infants, have demonstrated that as much as 74% of the iron in the formula presently under study may be absorbed. Other clinical reports have also pointed out that large quantities of iron are absorbed by iron-deficient infants fed a formula containing ferrous iron (9, 10). The gut lumen contains nondietary citrate (2, 4, 11) as well as organic acids, peptides, and amino acids which arise from digested food—these substances render ferrous phosphate soluble and dialyzable.

ACKNOWLEDGMENT

The authors gratefully acknowledge the cooperation of Doctor Bruce Johnson in the preparation of the Fe^{59}-labeled product used in this study.

REFERENCES

(1) Allan, J. E. The Oxidation of Ascorbic Acid in Dairy Products. J. Dairy Research, 17: 54. 1950.

(2) Carlson, A., and Hollunger, G. The Effect of Vitamin D on the Citric Acid Metabolism. Acta Physiol. Scand., 21: 317. 1954.

(3) Gorten, M. K., Workman, J. B., and Hepner, R. A Quantitative Study of Absorption of Labeled Iron in Premature Infants. Personal communication. 1961.

(4) Harrison, H. E., and Harrison, H. C. Studies with Radiocalcium: The Intestinal Absorption of Calcium. J. Biol. Chem., 188: 83. 1951.

(5) Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical Physiological Chemistry. McGraw-Hill Book Co., Inc., New York. 1954.

(6) Josephs, H. W. Absorption of Iron as a Problem in Human Physiology; A Critical Review. Blood, 13: 1. 1958.

(7) King, R. L., Luick, J. R., Litman, I. I., Jennings, W. G., and Dunkley, W. L. Distribution of Natural and Added Copper and Iron in Milk. J. Dairy Sci., 42: 780. 1959.

(8) Mackay, Helen M. M. Anaemia in Infancy: Its Prevalence and Prevention. Arch. Disease Childhood, 3: 117. 1928.

(9) Marsh, A., Long, H., and Stierwalt, E. Comparative Hematologic Response to Iron Fortification of a Milk Formula for Infants. Pediatrics, 24: 404. 1959.

(10) Schulz, J., and Smith, N. J. A Quantitative Study of the Absorption of Iron Salts in Infants and Children. Am. J. Diseases Children, 95: 120. 1958.

(11) Steenbock, H., and Bellin, S. A. Vitamin D and Tissue Citrate. J. Biol. Chem., 205: 985. 1953.

(12) Van der Waarden, J. Chemical and Physical Investigations on Dairy Products. Elsevier Publ. Co., New York. 1947.

INFLUENCE OF THE STAGE OF LACTATION AND ENVIRONMENTAL TEMPERATURES ON THE SALT BALANCE OF MILK[1]

T. H. KAMAL,[2] H. D. JOHNSON, AND A. C. RAGSDALE
Department of Dairy Husbandry, University of Missouri, Columbia

SUMMARY

Salt balance in milk was determined under controlled climatic laboratory conditions with two groups of six nonpregnant Holstein cows at comfort temperature (65° F.) and heat (80 and 90° F.). Each group consisted of two early-, mid-, and late-lactation cows. The two groups were alternately exposed (switch-back design) at 2-wk. intervals to comfort temperature (65° F.) and heat (80 or 90° F.) in the following successive temperatures: 65, 80, 65, 90, 65, and 90° F.

At 65° F. the average values for salt balance, i.e. (citric acid + phosphorus) − (calcium + magnesium), phosphorus, magnesium, and milk yield were, respectively, 0.55 Mol. × 10^{-3}/100 ml., 88 mg/100 ml., 10.9 mg/100 ml., and 36 lb/day, and at 80° F. they were, respectively, 0.29 Mol. × 10^{-3}/100 ml., 82 mg/100 ml., 10.7 mg/100 ml., and 33 lb/day.

High temperatures depressed citric acid and calcium contents during early lactation. The values at 65, 80, 65, and 90° F. were 181, 159, 150, and 148 mg/100 ml., respectively, for citric acid, and they were 107, 105, 104, and 103 mg/100 ml., respectively, for calcium.

Heat caused no marked change in sodium and sodium/potassium ratio, but it decreased potassium in all lactation periods.

With the progress in the stage of lactations at comfort temperature (65° F.), the early-, mid-, and late-lactation groups decreased exponentially in salt balance, citric acid, potassium, and milk yield. The percentage decline per week for the three lactation groups in sequence were 1.3, 5.8, and 12.2 for salt balance; 1.4, 1.1, and 2.1 for citric acid; 0.5, 0.6, and 1.9 for potassium; and 4.2, 4.6, and 6.0 for milk yield. Sodium and sodium/potassium ratios consistently increased at all lactation periods, with average rates of 2.2 and 2.9% per week, respectively.

Phosphorus and magnesium declined during early- and mid-, and increased during late-lactation periods. Calcium decreased during early lactation and increased at later stages of lactation.

The variations in the levels of the mineral constituents in milk is of a profound importance from the nutritional standpoint as well as in the successful handling of milk in dairy plants. Slight alterations in the relative proportions of some of these constituents may lead to serious trouble in cheese and evaporated milk manufacture. The troublesome heat coagulation of milk in the sterilizing process of evaporated milk manufacture as a result of relatively low citrates and phosphates in proportion to calcium and magnesium contents was suggested earlier (23). The calcium-ion concentration and colloidal phosphate content of milk were also shown to be the chief factors in the problem of heat coagulation (20). The heat stabilizers used in the commercial manufacture of

Received for publication January 1, 1961.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 2250. Approved by Director.

[2] Present address: UT-AEC Agricultural Research Laboratory, Box 1067, Oak Ridge, Tennessee.

evaporated milk are confined almost exclusively to the phosphate and citrate group and it is sometimes necessary to employ disodium phosphate to control the heat stability of evaporated milk to successfully manufacture the product (9). In cheese manufacture, on the other hand, the formation of a proper curd is achieved with milk that contains high calcium content (16, 26).

Many factors can bring about changes in the salt balance of milk by altering the calcium, magnesium, citrate, and phosphate contents of milk. The period of lactation and the season of the year are believed to be the most important factors responsible for such differences. No attempts, however, were undertaken to study the effect of each of these two factors separately and without the interference of the gestation on the mineral composition of milk.

The objective of this study was to investigate the effect of stage of lactation, under controlled comfort environment (65° F., 50% R.H.)[3] in nonpregnant cows, on milk yield, calcium, magnesium, phosphorus, citric acid, sodium, and potassium contents, as well as on the salt balance and sodium/potassium ratio of milk. The changes in the aforementioned constituents under the controlled environmental temperatures (65, 80, and 90° F.) also have been studied. Although the term minerals refers to the inorganic elements, the citric acid in milk was included in this term, due to its association with minerals in various phenomona.

MATERIALS AND METHODS

1. Animals and management. The experimental animals in this study consisted of 12 lactating Holstein cows. They were divided into two groups, (A and B), each consisting of two early-, two mid-, and two late-lactation cows. All animals were in their declining phase of milk production. The early-, mid-, and late-lactation animals were approximately in their 2nd, 5th, and 8th mo. of lactation, respectively. They were chosen in this way, so that by the end of the experimental period (3 mo.) a fair representation of the whole declining phase of the stage of lactation would be obtained. The animals were not bred, in order to eliminate the pregnancy effect in this study. Two cows, however, were in their 1st mo. of gestation when the experiment started. The influence of gestation in these two cows was of minor importance, since this experiment lasted only 3 mo.

The animals were kept in two separate, independently controlled climatic chambers. Alfalfa hay was fed in the form of fine-ground pellets. Cows were fed a regular type of milking ration of concentrates and beet pulp, the quantity of which varied with the milk production. Alfalfa pellets and water were provided ad libitum. Pellets were used for the purpose of controlling feed quality and obtaining accurate measures of feed intake.

2. Treatments. For the entire experiment of 12 wk., the temperature was constantly maintained in one chamber at 65° F., 50% relatively humidity. The other chamber was maintained at 80° F., 50% relative humidity during the

[3] The comfort zone is defined in the Missouri Environmental Physiology Series (I-LV) (1948-1959).

first 6 wk. of the experiment and at 90° F., 40% relative humidity during the rest of the experimental period (6 wk.). Special switch-back-type experimental design was used in exposing the animals to the high temperatures of 80 and 90° F. For example, Group A was first exposed to 65° F. for 2 wk., then to 80° F. for another 2 wk., then was switched back to 65° F. for 2 wk., and so on. This design was applied so that each cow would serve as its own control for the temperature study and, thus, individual variability would be minimized. By using such design it was also possible to separate the value obtained at 65° F. from those at high temperatures.

3. Sampling. Representative milk samples were taken from each cow individually on the evening, morning and evening, and morning of the ninth, tenth, and eleventh days, respectively, of each treatment period (2 wk.). Milk samples were refrigerated and no preservatives were added. Chemical analyses were undertaken on the last day of sampling (11th day of treatment period). The milk samples of each cow were mixed together in proportion to their corresponding milk yields. This system provided a two-day composite sample of milk from each cow during the 2nd wk. of each treatment at each of the three studied stages of lactation.

4. Methods of analyses. Calcium and magnesium were determined by the method of Kamal (11). Total phosphorus was determined by transferring a 5-ml. milk sample to a 100-ml. volumetric flask which was made to volume with distilled water. One milliliter of diluted milk was transferred to a 200- by 25-mm. test tube and 0.4 ml. concentrated sulfuric acid, followed by 0.5 ml. 70% perchloric acid, were added. The contents were heated over a very low flame of microburner for about 20 min., until the contents became colorless. The digested solution was cooled and transferred to a 25-ml. volumetric flask and was analyzed by the method of Fiske and Subbarrow (6). The optical density was read on the Coleman Junior Spectrophotometer at 600 mμ. Citric acid was determined by the method of Babad and Shtrikman (3). A 15% trichloracetic acid solution for protein precipitation and a Coleman Junior Spectrophotometer were used in the citric acid determination. Standard flame photometric procedure was used for sodium and potassium determination on the TCA-filtrate of milk. Salt balance was computed by subtracting the sum of calcium plus magnesium from the sum of phosphorus plus citric acid, each expressed as mol. per 100 ml. The results of salt balance were expressed as mol. $\times 10^{-3}$ per 100 ml. milk.

5. Graphic and statistical analysis of data. To study the effect of stage of lactation on the yield and composition of milk in this study, the average 65° F. milk yield and constituents were arranged together. Lactation curves for the fortnight averages are shown in Figures 1 and 2. The data were plotted on semilogarithmic paper and the exponential regression equation $Y = a.e^{bx}$ was used to provide an equal comparison between the rates of changes of all characteristics studied with the advance in lactation periods. A characteristic of b value, for example -0.05, means that this component declines at a rate of 5% per week with the advance in lactation period during the experimental period. These regressions are presented in Table 1. The values of 80 and 90° F. were

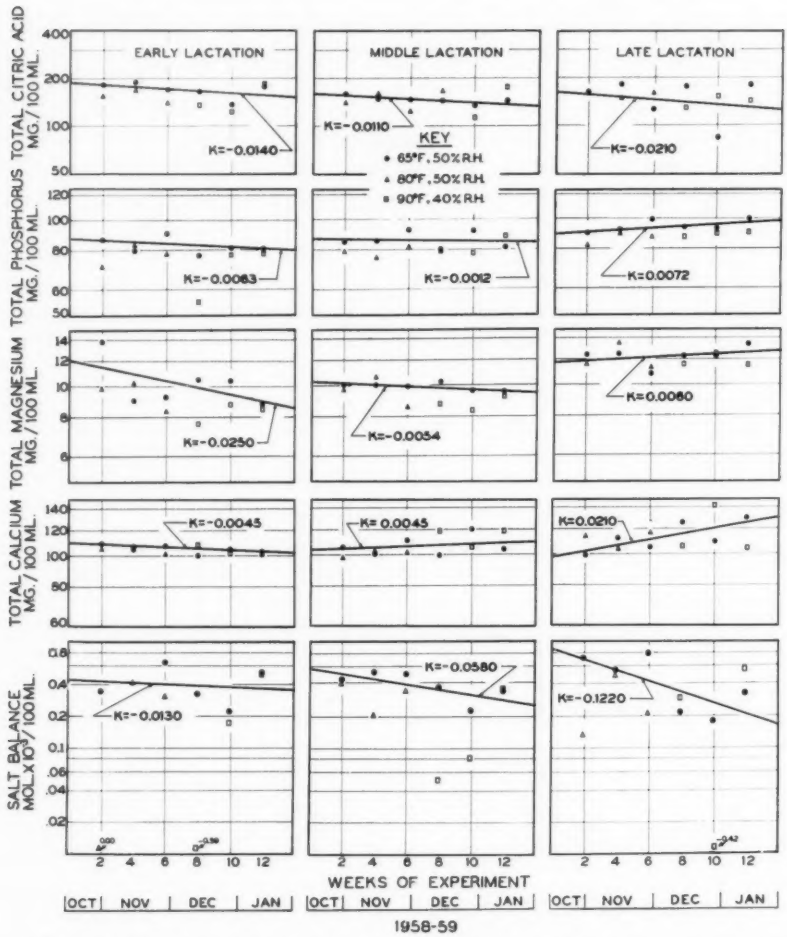


FIG. 1. Effect of advancing stage of lactation on salt balance in milk. Differences found in absolute values between the early, mid, and late stages of lactation are due to the changing composition (different cows) which make up the average values at each stage of lactation.

plotted on the graphs, however, to show their deviations from the lactation curves. Correlations between the weeks of the experiment and every constituent at early, mid, or late lactation are presented in Table 2.

To study the effect of environmental temperature on the yield and composition of milk, the values of the 2nd, 4th, and 6th wk. of the experiment at 65° F. were compared with the corresponding values at 80° F. The same procedure was undertaken for the values at the 8th, 10th, and 12th wk. of the experiment for 65 and 90° F. temperatures. The results are presented in Figure 3. The statistical differences, in the means of every constituent, between 65 and 80° F.

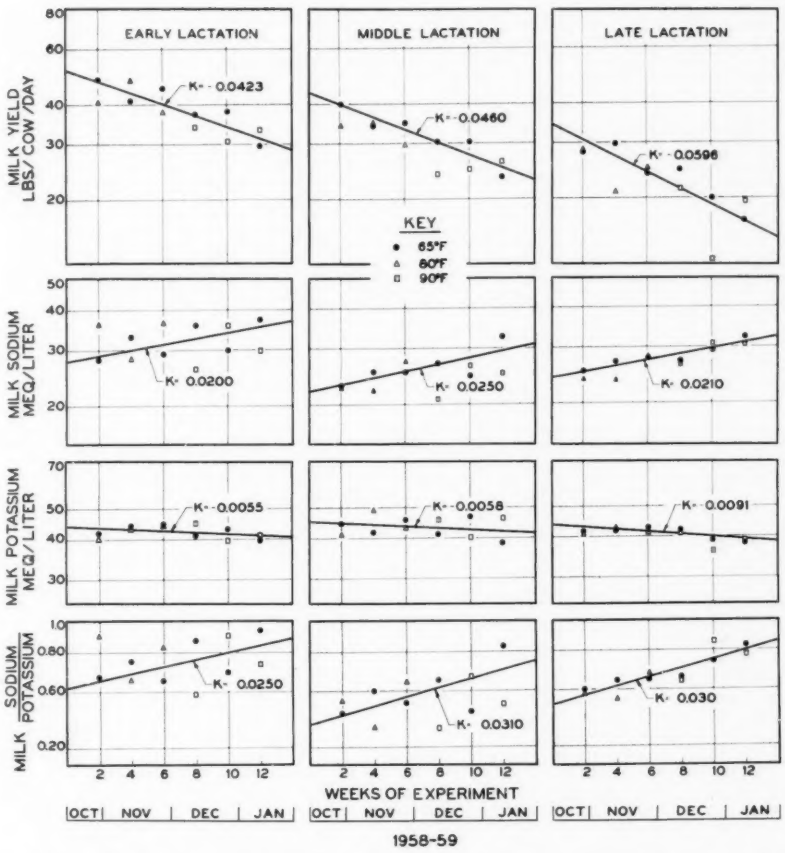


FIG. 2. Effect of advancing stage of lactation on sodium, potassium, sodium potassium ratio, and milk yield. Differences found in absolute values between the early, mid, and late stages of lactation are due to the changing composition (different cows) which make up the average values at each stage of lactation.

or between 65 and 90° F. during early, mid, or late lactation are shown (Table 3). Since each of Groups A and B had only one average at 80 and 65° F., respectively (the 4th wk. of the experiment), all values of the 4th wk. were multiplied by two. The same procedure was applied for the 90° F. treatment by multiplying the 10th-wk. values by two. Statistical analyses were undertaken by the Statistical Department on the electronic computer Burroughs E 102.

RESULTS

The influence of stage of lactation. Citric acid content: Figure 1 shows that at a comfort temperature (65° F.) Group A (2nd, 6th, and 10th wk. of the ex-

TABLE 1

The exponential regressions of milk constituents on advancing lactation period at 65° F. in cows at early, mid, and late lactations

Items	Regression coefficient b			Intercept a		
	Early	Mid	Late	Early	Mid	Late
Milk yield	−0.042	−0.046	−0.060	3.95	3.78	3.55
Total citric acid	−0.014	−0.011	−0.021	5.22	5.07	5.15
Total phosphorus	−0.006	−0.001	0.007	4.46	4.46	4.50
Total calcium	−0.005	0.005	0.021	4.96	4.64	4.58
Total magnesium	−0.025	−0.005	0.006	2.49	2.33	2.47
Salt balance	−0.013	−0.058	−0.122	−0.83	−0.53	−0.06
Sodium	0.020	0.025	0.021	3.32	3.09	3.19
Potassium	−0.006	−0.006	−0.009	3.79	3.81	3.78
Sodium/potassium	0.025	0.031	0.030	−0.47	−0.72	−0.59

TABLE 2

Correlation between milk constituents and the advance in lactation period at 65° F. in cows at early, mid, and late lactations

Items	Correlation coefficient r			D.F.
	Early	Mid	Late	
Milk yield	−0.56*	−0.70**	−0.57*	11
Total citric acid	−0.30	−0.56*	−0.20	11
Total phosphorus	−0.28	−0.04	0.32	11
Total calcium	−0.52	0.18	0.59*	11
Total magnesium	−0.50	−0.21	0.25	11
Salt balance	−0.07	−0.73**	−0.74**	11
Sodium	0.44	0.72**	0.68**	11
Potassium	−0.34	−0.26	−0.45	11
Sodium/potassium	0.50	0.60**	0.81**	11

* Statistically significant at $P > 0.05$.
** Statistically significant at $P > 0.01$.

TABLE 3

Test of significance between the means (65 vs. 80° F., 65 vs. 90° F.) of milk yield and milk constituents at various stages of lactation

Items	t Values between 65 and 80° F. averages				t Values between 65 and 90° F. averages			
	Early	Mid	Late	Total	Early	Mid	Late	Total
Milk yield	0.10	0.99	1.33	0.81	0.93	1.55	1.34	1.51
Total citric acid	1.75*	1.06	0.73	1.96*	0.48	0.15	0.64	0.28
Total phosphorus	1.49	2.34**	2.07*	2.96***	1.23	1.38	1.56	1.90*
Total calcium	0.77	1.08	0.77	0.36	0.07	0.17	0.55	0.29
Total magnesium	1.06	0.14	0.75	0.14	2.67**	1.74	1.06	1.98*
Salt balance	1.87*	4.09***	2.99***	4.56***	1.08	1.88*	0.91	1.95*
Sodium	0.49	1.15	1.67	0.40	0.51	1.53	0.11	0.88
Potassium	0.70	0.94	0.45	0.16	0.45	0.42	0.81	0.88
Sodium/potassium	0.69	1.13	1.34	0.27	0.23	0.83	0.82	0.29

* Statistically significant at $P > 0.10$.
** Statistically significant at $P > 0.05$.
*** Statistically significant at $P > 0.01$.
Degrees of freedom are 15 for early-, mid-, or late-lactation groups and 47 for total lactations.

periment) and Group B (4th, 8th, and 12th wk.) demonstrated a consistent decline in citric acid content with the advance in lactation period. This was true in early, mid, and late stages of lactation. The decline, however, was more pronounced at late- than at early- or mid-lactation periods. The exponential regressions (Table 1) show that the late-lactation group of cows declined with a rate of 2.1% per week, whereas the mid- and early-lactation cows declined at a rate of 1.1 and 1.4% per week, respectively. Table 2 shows that the correlation between the weeks of lactation and the citric acid content within each group was not statistically significant, except in mid lactation.

Total phosphorus content. During the early period of lactation (2nd to 12th wk. of lactation) the total phosphorus content decreased about 0.6% per week, as shown in Figure 1. No marked change, or possibly a slight decline, was observed in the phosphorus content of milk during the middle part of lactation period. This was followed by a continuous rise of about 0.7% per week in the last 3 mo. of lactation (Table 1).

Total magnesium content. Similar changes in magnesium to that of phosphorus were observed with advancing lactation, either in early-, mid-, or late-lactation periods (Figure 1). The decline in magnesium content during early and mid lactations, however, was much faster (2.5 and 0.5% per week, respectively) than that of phosphorus. The rate of increase, thereafter, in magnesium was close to that of phosphorus content. No statistically significant correlation between the stage of lactation and either the phosphorus or magnesium contents, however, was obtained in these data (Table 2).

Total calcium content. In early lactation a decline of about 0.5% per week took place in calcium content, as shown in Figure 1. This was followed by an increase of 0.5% per week during mid lactation, and a statistically significant rise of 2.1% per week during the last 3 mo. of lactation period (Tables 1 and 2).

Salt balance. A declining trend was observed in salt balance with advancing lactation, as shown in Figure 1. This effect of stage of lactation on salt balance was statistically significant in mid- and late- but not in early-lactation periods (Table 2). The rate of decline was pronounced as the stage of lactation progressed. The exponential regression coefficients (Table 1) show that milk salt balance decreased about 1.3, 5.8, and 12.2% per week in early-, mid-, and late-lactation periods, respectively.

Milk yield. With the advance in lactation period a statistically significant decline in milk yield was demonstrated in early, mid, and late stages of lactation (Tables 1 and 2). This decline was more pronounced at late- than at early- or mid-lactation periods (Figure 2). The rate of decline was about 4.2, 4.6, and 6.0% per week in early-, mid-, and late-lactation periods, respectively.

Sodium content. Figure 2 shows that with the advance in lactation either in the early, mid, or late periods, the sodium concentration in milk increased exponentially in both Groups A and B. The data in Table 1 show the rate of increase was about 2.0, 2.5, and 2.1% per week, for early-, mid-, and late-lactation periods, respectively. This increase was statistically significant for the mid- and late-lactation periods, as shown from the correlation coefficients in Table 2.

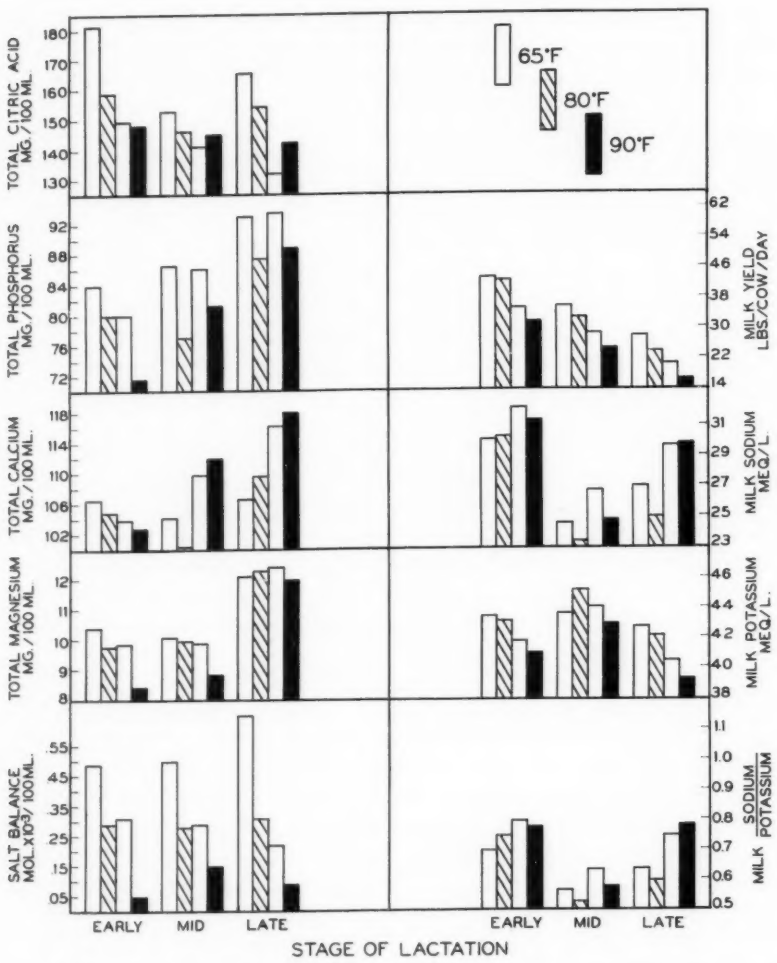


FIG. 3. Influence of environmental temperature on milk yield, mineral composition, salt balance, and Na/K in milk.

Potassium content. A slight but continuous decline in potassium content took place throughout the entire lactation period (Figure 2). Such decline was more pronounced at late than at early or mid periods of lactation (Table 1). No statistically significant correlation, however, was obtained between the advance in lactation and the potassium content of milk (Table 2).

Sodium/potassium ratio. Figure 2 shows that the sodium/potassium ratio followed the same trend of sodium content, with advancing lactation demonstrating a constant rise throughout the entire lactation period (Table 1). The posi-

tive correlations between sodium/potassium ratio and the weeks of lactation were statistically significant in the mid and late stages of lactation (Table 2).

The influence of environmental temperature. Citric acid content. Figure 3 shows that the average of citric acid content was higher at 65° F. than the corresponding average at 80° F. This was true for early-, mid-, and late-lactation periods. The difference between these temperature levels in citric acid content was statistically significant at early lactation and not at mid or late lactations. When all observations were pooled together the difference was found significant (Table 3). In the second treatment (65° F., 90° F.) the citric acid content decreased slightly with high environmental temperature only at early lactation and increased above the values of 65° F. at mid- and late-lactation periods. The difference between low- and high-temperature values of citric acid content in this second treatment was, however, insignificant.

Total phosphorus content. At high environmental temperature (80° F.) the total phosphorus content in milk was markedly depressed. The difference between 65 and 80° F. values was statistically significant in mid and late lactations as well as in the combined data of all lactations (Table 3). Such influence of heat was, however, slightly significant in the second treatment when temperature was raised to 90° F.

Total magnesium contents. Results similar to those of phosphorus were obtained in magnesium content of milk. The values of magnesium content at either 80 or 90° F. were lower, though not statistically significant, than those at 65° F. throughout most of the lactation period.

Total calcium content. It is shown in Figure 3 that at high environmental temperature, 80 or 90° F., the calcium content in milk was lower than that at 65° F. during early lactation. It increased thereafter at high temperatures during the rest of lactation period. The difference between low and high temperature in calcium content, however, lacked statistical significance (Table 3).

Salt balance. At high environmental temperatures, 80 or 90° F., the salt balance was markedly lower than the corresponding values of 65° F., as shown in Figure 3. Such influence of heat on salt balance of milk was statistically significant (Table 3). Similarly to phosphorus content the depressing effect of heat on salt balance was less pronounced in the second treatment (65 vs. 90° F.) than in the first treatment.

Milk yield. In both treatments (65 × 80° F. and 65 × 90° F.) milk yield was lower at high than at low temperatures (Figure 3). The depression in milk yield due to heat was more pronounced as the animals advanced in lactation. For example, at 90° F. milk yield decreased only 10% in early lactation, whereas it decreased about 15 and 20%, respectively, in mid and late lactations. No statistically significant difference was obtained between the levels of milk yield at low and high temperature (Table 3), possibly due to the small number of observations.

Sodium content. No consistent response of sodium concentration to heat can be noted in either of the two treatments. Figure 3 shows that at 80° F. the sodium content was slightly higher than that at 65° F. during early lactation

period. It was lower, however, during mid and late lactations than the corresponding values of 65° F. In the second treatment, the sodium content at 90° F. was lower in early and mid and slightly higher in late lactation than the values of 65° F.

Potassium content. All the potassium content values obtained at high environmental temperatures were lower than their corresponding values at 65° F. This was true in early, mid, and late lactations (Figure 3). No statistically significant difference, however, was obtained between the cold and heat treatments in regard to the potassium content of milk.

Sodium/potassium ratio. Results similar to those of sodium content were obtained in Na/K ratio environmental temperature effect.

DISCUSSION

It is indicated from the above results that the mineral composition of milk undergoes well-marked changes as the period of lactation advances. The changes observed in this investigation on nonpregnant cows are in agreement with those obtained from presumably pregnant cows (1, 5, 8, 23, 27). It seems, therefore, that pregnancy, or the great demand of minerals for foetus growth, is not a causative element for the observed trends of mineral constituents with advancing lactation. Moreover, the decline in milk volume with advancing lactation does not seem to be responsible for these trends, since the mineral constituents do not have similar directional trends to each other with the advance in lactation period.

The suggested decrease in prolactin, growth hormone, and thyrotropin due to low estrogen secretion (25) in these nonpregnant cows, however, might contribute to the changes observed in the mineral constituents of milk with advancing lactation. Few studies, however, have been undertaken in regard to the hormonal influence on milk composition in general and its mineral constituents in particular (2, 4, 7, 17, 22).

The statistically significant decline in salt balance with the advance in lactation period in this study indicates that the difference between the anions and cations, i.e. (citric acid + phosphorus) − (calcium + magnesium) decreases as the cow advances in lactation period. According to the salt balance theory, milk obtained from cows at early lactation is expected to have higher protein stability than that obtained at later periods of lactation. White and Davies (28), however, found little relation between heat stability of milk and stage of lactation of the cow, and later they (29) showed that renneting time was increased as lactation period advanced, in contrast to what would be expected in the present study.

The effect of environmental temperature on the mineral constituents and salt balance of milk as shown from the above results indicates that milk yield, total phosphorus, and total magnesium decreased at high environmental temperatures, 80 and 90° F. Thyroxine, which is generally known to increase milk yield and phosphorus content of milk (22), is depressed in cows at high environmental temperature (10, 13, 19). The above results may be attributed to a low

thyroid gland activity of the cows under hot conditions. These results are in agreement with those of Nickerson (18) on total phosphorus and total magnesium, and by Ragsdale *et al.* (21) on milk yield.

Citric acid content in this trial was significantly lower at high than at low temperatures during early-lactation period, whereas no further consistent response to heat was observed during mid- and late-lactation periods. The total calcium content of milk was depressed at high temperatures (80 and 90° F.) during the first half of the lactation period, whereas the reverse was true during the rest of the lactation period. It seems from these results that the stage of lactation modifies to a great extent the temperature influence on citric acid and calcium contents of milk. This may, however, explain the contradiction between the results obtained by Ellenberger *et al.* (5) and those obtained by Nickerson (18) in their study of the seasonal effects on milk calcium content. Kleiber (15) has indicated the existence of citric acid cycle in the mammary gland of intact dairy cow, and Terner (24) showed that the formation of citric acid is more active than its oxidation in the mammary gland homogenates of the guinea pigs, and he suggested that this accounted for the accumulation of citric acid in mammary tissue and its appearance in milk. The question may arise as to what influence various hormones have on the citric acid cycle in the mammary gland of the cow and whether the decrease in thyroxine secretion at high temperature, and possibly with advancing lactation, may account for the citric acid results observed in this investigation.

Salt balance showed a statistically significant decline at 80 and 90° F., indicating a smaller difference between anions (citrates + phosphates) and cations (calcium + magnesium) in heat than in cold. According to the salt balance theory this result suggests a low protein stability of milk at high temperature. This contradicts the general phenomenon of heat coagulation and long renneting time troubles in dairy manufacture, which are most prevalent during winter and spring or summer, respectively (14, 23). It has been mentioned earlier in this discussion that salt balance was high at early lactation and decreased significantly with advancing lactation. Since most of the cows freshen in spring and summer it seems that the depressing effect of heat on salt balance may be overweighed by the boosting effect of early-lactation period on salt balance, with resultant over-all increase in milk salt balance during summer. This may account for the troublesome increase in renneting time of cheese manufactured during the spring and summer seasons.

It is rather interesting to find that, though heat significantly causes natruresis, low urinary potassium excretion, and increase of urinary Na/K ratio, as well as insignificant inversed effects on blood electrolytes in dairy heifers (12), milk sodium and Na/K ratio showed no marked response to heat treatments (80 and 90° F.). The last author attributed his results to high glucocorticoids and low mineralocorticoids secretion in dairy animals that were under heat stress. It seems, however, that corticoids have no significant effect on milk electrolytes (7). The decline in potassium content of milk at high temperatures may be due

to the decline in roughage consumption, which is the main source of potassium in the feed.

REFERENCES

(1) ALLEN, L. A. The Mineral Constituents and Citric Acid Content of Milk. J. Dairy Research, 3: 1. 1931.

(2) ASKER, A. A., RAGAB, M. T., AND KAMAL, T. H. The Effect of Feeding Thyroprotein on the Composition of Milk of Egyptian Buffaloes. Indian J. Dairy Sci., 7: 36. 1954.

(3) BABAD, J., AND SHTRIKMAN, N. The Estimation of Citric Acid in Dairy Products. J. Dairy Research, 18: 72. 1951.

(4) BARTLETT, S., AND HUTTON, J. B. Some Hormone-Induced Changes in Milk Composition. Intern. Dairy Congr., 1959, 1: 135. 1959.

(5) ELLENBERGER, H. B., NEWLANDER, J. A., AND JONES, C. H. Variations in the Calcium and Phosphorus Contents of Cow's Milk. Vermont Agr. Expt. Sta., Bull. 556. 1950.

(6) FISKE, C. H., AND SUBBARROW, Y. The Colorimetric Determination of Phosphorus. J. Biol. Chem., 66: 375. 1925.

(7) FLUX, D. S., FOLLEY, S. J., AND ROWLAND, S. J. The Effect of Adrenocorticotrophic Hormone on the Yield and Composition of the Milk of the Cow. J. Endocrinol., 10: 333. 1954.

(8) HOLWEDRA, K. Variaties in het citroenzuurgehalte van melk, Gelevard aan Zuivelfabrieken in Friesland. Netherlands Milk Dairy J., 8: 115. 1954.

(9) HUNZIKER, O. F. Condensed Milk and Milk Powder. 7th ed. La Grange, Illinois, 1949.

(10) JOHNSON, H. D. Effect of Rising Environmental Temperatures (35-95° F.) on Thyroid I^{131} Activity of Holstein, Brown Swiss, and Jersey Heifers. J. Dairy Sci., 41: 744. 1958.

(11) KAMAL, T. H. Complexometric Titration of Calcium and Magnesium in the Presence of Phosphate in Milk and Blood Plasma. J. Agr. Food Chem., 8: 156. 1960.

(12) KAMAL, T. H. Metabolic Reactions During Thermal Acclimation at 50° and 80° F. and During Thermal Stress (35° to 95° F.) in Dairy Animals. Dissertation Abstr., 21: 1. 1960.

(13) KAMAL, T. H., JOHNSON, H. D., AND RAGSDALE, A. C. The Effect of Long Exposure of Environmental Temperatures of 50° and 80° F. on Glutathione, BEI^{131}, and Growth Rate of Dairy Calves. Missouri Agr. Expt. Sta., Research Bull. 710. 1959.

(14) KAMAL, T. H., JOHNSON, H. D., AND RAGSDALE, A. C. Personal communication with various American dairy plants. 1959.

(15) KLEIBER, M., AND BLACK, A. L. Tracer Studies on Milk Formation in the Intact Dairy Cow. Atomic Energy Commission Report TID-7512, p. 395. 1956.

(16) KOVALENKO, M. S., AND MOISEEV, A. S. Use of Ion Exchange in the Manufacture of Dairy Products. Doklady Vsesoyuz. Konf. Molch. Delu 1958, pp. 444 (D.S.A., 21: 171. 1959).

(17) MOSIMANN, VON W. Vergroberung der kernvolumina in der Parathyreoidea und vermehrte Kalzium-ausscheidung in der milch bei ziegen nach kunstlicher auslosung der Laktation Durch Oestrogene. Separat-Abdruck aus dem, Schweiz. Arch. Tierheilk., 97. 1955.

(18) NICKERSON, T. A. Chemical Composition of Milk. J. Dairy Sci., 43: 598. 1960.

(19) PREMACHANDRA, B. N., PIPES, G. W., AND TURNER, C. W. Variation in the Thyroxine-Secretion Rate of Cattle. J. Dairy Sci., 41: 1609. 1958.

(20) PYNE, G. T., AND MCHENRY, K. A. The Heat Coagulation of Milk. J. Dairy Research, 22: 60. 1955.

(21) RAGSDALE, A. C., WORSTELL, D. M., THOMPSON, H. J., AND BRODY, S. Influence of Temperature, 50° to 0° F. and 50° to 95° F., on Milk Production, Feed and Water Consumption and Body Weight in Jersey and Holstein Cows. Missouri Agr. Expt. Sta., Research Bull. 449. 1949.

(22) SENGUPTA, J., AND CHANDA, R. Some Factors Affecting the Composition of Cow's Milk and Buffalo's Milk. Intern. Dairy Congr. 1959, 1: 147. 1959.

(23) SOMMER, H. H., AND HART, E. B. Heat Coagulation of Evaporated Milk. Wisconsin Agr. Expt. Sta., Research Bull. 67. 1926.

(24) TERNER, C. The Metabolism of Citric Acid in the Mammary Gland. I. The Effect of *p*-Nitrophenol on the Synthesis and Oxidation of Citric Acid by Homogenates of the Mammary Gland. Biochem. J., 60: 88. 1955.

(25) TURNER, C. W. The Experimental Induction of Growth of the Cow's Udder and the Initiation of Milk Secretion. Missouri Agr. Expt. Sta., Research Bull. 697. 1959.

(26) VERMA, I. S., AND GEHRKE, C. W. The Enzymic Phase of the Coagulation of Milk by Rennet. Intern. Dairy Congr., 1959, 2: 648. 1959.

(27) WHITE, J. C. D., AND DAVIES, D. T. The Relation Between the Chemical Composition of Milk and the Stability of the Caseinate Complex. I. General Introduction, Description of Samples, Methods and Chemical Composition of Samples. J. Dairy Research, 25: 236. 1958.

(28) WHITE, J. C. D., AND DAVIES, D. T. The Relation Between the Chemical Composition of Milk and the Stability of the Caseinate Complex. III. Coagulation by Rennet. J. Dairy Research, 25: 267. 1958.

(29) WHITE, J. C. D., AND DAVIES, D. T. The Relation Between the Chemical Composition of Milk and the Stability of the Caseinate Complex. IV. Coagulation by Heat. J. Dairy Research, 25: 281. 1958.

REPRODUCTIVE CAPACITY OF DAIRY BULLS. II. GONADAL AND EXTRA-GONADAL SPERM RESERVES AS DETERMINED BY DIRECT COUNTS AND DEPLETION TRIALS; DIMENSIONS AND WEIGHT OF GENITALIA[1]

J. O. ALMQUIST AND R. P. AMANN

Dairy Breeding Research Center, Department of Dairy Science
The Pennsylvania State University, University Park

SUMMARY

Extra-gonadal sperm reserves (EGR) determined by direct counts averaged 69.4 billion for 15 sexually rested (SR) bulls, 73.5 billion for 15 sexually rested bulls depleted just before slaughter (SR-D), and 68.0 billion for five bulls ejaculated six or seven times per week for at least 6 wk. prior to slaughter (6-7 ×). While total EGR, which included an average of 38.5 billion sperm collected on the day of slaughter from SR-D bulls and 10.5 billion from 6-7 × bulls, did not vary significantly among the three groups, sperm distribution was significantly different. Sperm counts of the caput, corpus, and cauda epididymis averaged 19.4, 4.7, and 37.6 billion for SR bulls, 16.2, 3.0, and 13.7 billion for SR-D bulls, and 22.6, 5.2, and 26.4 billion for 6-7 × bulls. Depletion removed 52.8 ± 2.3% of the total EGR of SR-D bulls; although the cauda epididymis was nearly emptied, partial replenishment with sperm from the caput and corpus occurred during the 60- to 90-min. interval between the end of depletion and slaughter. Daily sperm output for the 6-7 × bulls averaged 4.9 billion for 6 wk. The sperm content of the cauda epididymis was 25% lower than if they had been sexually rested. The ductus deferens and ampulla of SR bulls contained only 7.6 billion sperm. When corrected for preslaughter treatment, the EGR for the 35, 76 ± 5-mo.-old bulls averaged 72.6 billion sperm. Preslaughter treatment did not affect gonadal sperm reserves. Fifty testes, averaging 374 g., contained 20.2 billion sperm, or 54.8 ± 1.0 million per gram. Testis sperm numbers were highly significantly correlated (+0.62) with testis weight. The highly significant correlation (+0.87) between EGR and total sperm removed in a depletion should permit estimation of EGR in living animals by depletion trials of about 20 ejaculates. Gonadal sperm reserves were not significantly correlated to epididymal sperm reserves in SR bulls. The independence of testicular sperm production and storage of sperm in the excurrent duct system is stressed. Genitalia dimensions and weight for 55 bulls are presented by breeds and ages.

The first indirect measurements of extra-gonadal sperm reserves (EGR) of bulls by depletion trials were reported by Walton and Edwards (27). They collected ten ejaculates over a 2-hr. period and obtained an average of 15 billion sperm from 14 bulls. Subsequently, Almquist and co-workers (2, 3, 15, 16) reported on more demanding depletion trials, in which 37 bulls averaged 37.3 billion sperm (average of 24 ejaculates for 80 trials). These authors agreed with Walton and Edwards that the number of sperm removed during depletion represented a minimum estimate of EGR. However, only by direct determinations of the EGR can its relation to depletion values be established.

Received for publication April 10, 1961.

[1] Authoried for publication on March 31, 1961, as paper No. 2545 in the journal series of The Pennsylvania Agricultural Experiment Station.

Direct measurements of sperm reserves in rams (10, 22, 24) and boars (17, 20) and small numbers of rabbits (9, 12, 19), rats (9), guinea pigs (9), and hamsters (11) have been reported since 1938. Not until 1956, however, were the first direct determinations of EGR in bulls published (3, 6, 21). Counts of gonadal sperm reserves in bulls have been presented in preliminary reports by Ortavant (21) and Almquist *et al.* (2), as well as in a thesis by Frederick (13).

A suitable technique for counting the number of sperm in the bovine reproductive system has been developed at this Station (5). Using this technique, the actual gonadal and extra-gonadal sperm reserves were determined for bulls slaughtered following sexual rest, depletion, or a period of ejaculation at high frequency. The results are compared to those obtained by indirect methods of measuring sperm reserves.

EXPERIMENTAL PROCEDURE

Thirty-five bulls 2 to 12 yr. of age were slaughtered following either (a) sexual rest for a minimum of 30 days (15 bulls), (b) depletion of EGR after sexual rest for at least 30 days (11 bulls) or after collection of one ejaculate per week (four bulls), or (c) collection of six or seven ejaculates per week for at least 6 wk. The bulls in the latter group were either ejaculated daily (one bull) or two ejaculates were collected each Monday, Wednesday, and Friday (four bulls). In the depletion trials, the first ejaculate was preceded by 2-min. restraint and one false mount; as a rule, false mounts were not used prior to subsequent ejaculations. In the third group, a similar procedure was used with two of the bulls on six ejaculates per week, whereas 2-min. restraint and three false mounts were used prior to each ejaculate with the other three bulls. These five bulls were collected at their regular frequency on the day of slaughter. The bulls were trucked 30 mi. to an abattoir and killed 60 to 90 min. after the last collection. Gross examination indicated that the genitalia were normal. Reproductive organ dimensions and weights were obtained 3 to 4 hr. after slaughter. Gonadal and extra-gonadal sperm reserves were determined by the methods outlined by Amann and Almquist (5).

RESULTS AND INTERPRETATION

Reproductive organ characteristics for the 35 experimental bulls and 20 additional bulls are summarized by breeds and ages (Table 1). For all 55 bulls, within-bull testis variation was very small; the left testis averaged 8.1 g. heavier than the right. Although confounded by breed differences, paired testes weight was highly significantly ($P < 0.01$) correlated to age (+0.51), but not to body weight (+0.15). Testis and epididymis weight also were highly significantly correlated (+0.82). Considering only 30 purebred Holsteins (excluding one 18-yr.-old), paired testes weight was significantly ($P < 0.05$) correlated with body weight (+0.42) and highly significantly with age (+0.53).

Table 2 characterizes the sexually rested (SR), depleted (SR-D), and frequently ejaculated (6-7 ×) groups of bulls, as well as their gonadal and extra-gonadal reserves (EGR). Table 3 presents pertinent correlation coefficients.

TABLE 1

Dimensions and weight of the reproductive organs of dairy bulls[a]

Portion of reproductive system	Holstein & Holstein-cross					Guernsey, Jersey, & Ayrshire					All breeds				
	25-48 mo.	49-72 mo.	73-96 mo.	97+ mo.	Av.	25-48 mo.	49-72 mo.	73-96 mo.	97+ mo.	Av.	25-48 mo.	49-72 mo.	73-96 mo.	97+ mo.	Av.
No. bulls	7	20	4	7	38	4	3	5	5	17	11	23	9	12	55
Age *(mo.)*	37	59	80	133	71	40	61	88	101	76	38	59	84	120	72
Weight *(lb.)*	1,864	2,081	2,046	2,006	2,023	1,578	1,445	1,672	1,708	1,620	1,760	1,998	1,838	1,883	1,899
Testis															
Weight *(g.)*	259	335	359	395	334	287	358	433	447	390	269	338	400	418	351
Volume *(ml.)*	256	317	351	377	323	274	341	414	430	373	264	320	386	400	339
Length *(cm.)*	11.3	12.7	12.7	13.1	12.5	12.2	12.8	13.5	13.4	13.0	11.6	12.7	13.2	13.2	12.7
Diameter *(cm.)*	6.5	7.1	7.4	7.4	7.0	6.6	7.1	7.5	7.5	7.2	6.5	7.1	7.4	7.5	7.1
Circumference *(cm.)*															
Length	28.7	31.4	31.7	32.8	31.2	29.8	31.9	34.1	34.4	32.7	29.2	31.5	32.9	33.5	31.7
Width	19.6	21.1	21.8	22.1	21.1	19.8	22.0	23.1	23.2	22.1	19.6	21.2	22.5	22.6	21.4
Epididymis															
Weight *(g.)*	27.0	35.0	38.3	40.4	34.8	28.9	38.6	41.2	44.6	38.9	27.7	35.5	40.0	42.2	36.1
Length *(cm.)*	20.1	20.8	20.8	22.3	21.0	21.1	22.7	22.8	23.8	22.9	20.4	21.0	21.8	23.0	21.6
Seminal vesicle															
Weight *(g.)*	54.6	78.2	78.9	80.9	75.8	40.0	50.0	87.3	86.3	74.4	49.8	74.5	83.6	83.2	75.4
Length *(cm.)*	12.1	13.4	14.9	13.7	13.4	9.8	11.6	13.8	14.0	12.5	11.2	13.2	14.3	13.8	13.1
Width *(cm.)*	2.9	3.2	3.2	3.0	3.1	2.7	2.8	3.5	3.6	3.2	2.8	3.2	3.5	3.3	3.2
Depth *(cm.)*	2.0	2.3	2.1	2.1	2.2	1.8	2.2	2.5	2.5	2.3	1.9	2.3	2.3	2.3	2.2
Cowper's gland															
Weight *(g.)*	5.2	6.5	7.1	6.0	6.2	5.2	5.7	6.8	8.4	6.7	5.2	6.4	7.0	7.0	6.4
Length *(cm.)*	2.9	3.4	3.8	3.5	3.4	3.4	3.2	3.3	3.6	3.4	3.1	3.4	3.5	3.5	3.4
Width *(cm.)*	2.0	2.2	2.3	2.2	2.2	1.8	2.1	2.1	2.1	2.0	2.0	2.2	2.2	2.2	2.1
Depth *(cm.)*	1.4	1.6	1.6	1.6	1.6	1.4	1.6	1.7	1.8	1.6	1.4	1.6	1.6	1.7	1.6
Ductus deferens length *(cm.)*															
Total	99.8	101.6	103.8	110.2	103.2	91.9	100.1	100.7	103.7	99.6	96.9	101.4	102.1	107.5	102.1
Ampulla	15.8	16.1	17.3	18.0	16.5	13.7	17.7	18.0	17.2	16.6	15.0	16.2	17.7	17.6	16.5
Penis															
Length *(cm.)*	95.4	97.4	103.1	106.4	99.3	91.6	95.4	97.1	93.2	94.4	94.0	97.1	99.8	100.9	97.8

[a] Thirty-one Holstein, eight crossbred Holstein, 14 Guernsey, two Jersey, and one Ayrshire.

[1] Thirty-one Holstein, eight crossbred Holstein, 14 Guernsey, two Jersey, and one Ayrshire.

TABLE 2

Gonadal and extra-gonadal sperm reserves of dairy bulls
(Mean ± S.E.)

Portion of tract (paired)	15 Sexually rested		15 Depleted		5 Frequently ejaculated	
	Sperm × 10^9	% of Total	Sperm × 10^9	% of Total	Sperm × 10^9	% of Total
Age *(mo.)*	80 ± 7		73 ± 8		72 ± 5	
Body weight *(lb.)*	1,866 ± 57		1,871 ± 97		1,972 ± 55	
Testis weight *(g.)*	384 ± 20		335 ± 14		398 ± 17	
Breeds						
Holstein	6		7		4	
Holstein-cross	2		4		0	
Guernsey	6		3		1	
Jersey	1		1		0	
Testis						
Total sperm	40.6 ± 2.4		37.1 ± 3.4		45.5 ± 2.9	
Sperm/g (× 10^6)	54.9 ± 2.7		52.4 ± 3.5		58.3 ± 5.9	
No. bulls	12		8		5	
Epididymis						
Caput	19.4 ± 1.5	28.1 ± 1.6	16.2 ± 1.4	22.6 ± 1.5	22.6 ± 1.9	33.2 ± 2.3
Corpus	4.7 ± 0.6	7.0 ± 0.9	3.0 ± 0.4	4.1 ± 0.3	5.2 ± 0.9	7.6 ± 1.4
Cauda	37.6 ± 2.5	54.3 ± 2.2	13.7 ± 2.0	17.6 ± 1.3	26.4 ± 2.3	38.7 ± 2.3
Total	61.8 ± 3.2	89.4 ± 1.2	32.9 ± 3.5	44.2 ± 2.4	54.2 ± 3.2	79.5 ± 2.0
Ductus deferens	2.5 ± 0.4	3.4 ± 0.5	0.6 ± 0.1	0.8 ± 0.2	1.4 ± 0.3	2.0 ± 0.3
Ampulla	5.1 ± 1.0	7.2 ± 1.3	1.5 ± 0.2	2.2 ± 0.2	1.9 ± 0.4	2.8 ± 0.7
Total in tract	69.4 ± 3.8		35.0 ± 3.6		57.5 ± 3.4	
Ejaculated	None		38.5 ± 3.5[a]	52.8 ± 2.3	10.5 ± 1.5[b]	15.7 ± 2.3
Total extra-gonadal reserves	69.4 ± 3.8		73.5 ± 6.2		68.0 ± 2.5	
Range	48.2 to 100.1		37.7 to 118.2		61.7 to 76.6	

[a] The preslaughter depletion trials averaged 19.1 ± 1.1 ejaculates (range 11 to 26) and contained 19.2 to 65.5 billion sperm.
[b] Two ejaculates were collected from four bulls and one ejaculate from the fifth bull on the day of slaughter.

TABLE 3

Correlation coefficients

Variables	Sexually rested bulls	Depleted bulls	All bulls
Number of comparisons	15	15	35
Extra-gonadal reserves and paired testes weight	0.454	0.448	0.357[a]
Extra-gonadal reserves and paired epididymis weight	0.793[b]	0.516[a]	0.560[b]
Extra-gonadal reserves and sperm in depletion		0.873[b]	
Extra-gonadal reserves and age	0.173	0.210	0.199
Extra-gonadal reserves and body weight	−0.193	0.340	0.182
Number of comparisons	30	30	70
Testis weight and epididymis weight	0.763[b]	0.890[b]	0.782[b]
Testis weight and sperm in caput epididymis	0.635[b]	0.554[b]	0.654[b]
Testis weight and sperm in epididymis	0.451[a]	0.676[b]	0.594[b]
Epididymis weight and sperm in epididymis	0.714[b]	0.661[b]	0.558[b]
Number of comparisons	24	16	50
Testis sperm and testis weight	0.650[b]	0.708[b]	0.616[b]
Testis sperm and sperm in caput epididymis	0.416[a]	0.651[b]	0.544[b]
Testis sperm and sperm in corpus epididymis	0.512[a]	0.800[b]	0.521[b]
Testis sperm and sperm in caput and corpus epididymis	0.560[b]	0.759[b]	0.648[b]
Testis sperm and sperm in cauda epididymis	−0.155	0.926[b]	0.267
Testis sperm and sperm in epididymis	0.141	0.907[b]	0.480[b]

[a] Significant at the 5% level.
[b] Significant at the 1% level.

Gonadal sperm reserves, determined for 25 of the 35 bulls, varied among the three treatment groups, but the differences were not significant. The differences were associated primarily with variation in testicular weight. This was shown by the highly significant correlation (+0.62) between testis weight and testis sperm, as well as the similarity among groups in the number of sperm per gram of testis. The 50 testes (374 ± 1 g.) averaged 20.2 ± 0.6 billion sperm, or 54.8 ± 1.0 million per gram. The correlation of +0.62 probably is too low to permit accurate estimation of gonadal sperm reserves from testicular weight in a heterogeneous group of bulls.

Total EGR ranged from 37.7 to 118.2 billion sperm and was not significantly different among the three groups of bulls. However, there were significant differences in sperm distribution associated with preslaughter treatment. In SR bulls, the cauda epididymis contained 37.6 billion sperm, or 54% of the total EGR, whereas the caput contained 19.4 billion sperm. The ductus deferens and ampulla together contained only 7.6 billion sperm—sufficient for one ejaculate. Thus, in SR bulls the major area of sperm storage is the cauda epididymis.

In SR-D bulls, on the other hand, the collection of 19.2 to 65.5 billion sperm just prior to slaughter (19.1 ± 1.1 ejaculates) resulted in a marked reduction in the sperm content of the cauda epididymis. The pattern of semen characteristics for these ejaculates was similar to that reported by Almquist and Hale (3). The 38.5 ± 3.5 billion sperm removed by depletion represented 85% of the total sperm content of the cauda epididymis, ductus deferens, and ampulla of SR bulls. These portions of the reproductive system contained an average

of 45.2 billion sperm in SR bulls, but only 15.8 billion in SR-D bulls. Thus, the majority of sperm initially present in the cauda epididymis was removed by depletion and approximately one-half of the sperm present in the cauda of SR-D bulls had moved in from more proximal portions of the epididymis.

The caput epididymis of SR bulls did not contain significantly more sperm than for SR-D bulls, but the SR-D bulls had a larger EGR. With an EGR of 73.5 billion sperm, the SR-D bulls should have averaged 20.6 billion sperm in the caput epididymis if 28.1% of their EGR was in the caput, as was true for SR bulls. The 16.2 billion sperm observed was only 79% of this value. This significant difference also suggests a rapid movement of sperm out of the caput epididymis as a result of depletion.

Since the correlation between EGR (including ejaculated sperm) and total sperm removed by depletion was highly significant (+0.87), depletion trials of approximately 20 ejaculates should permit accurate estimation of the EGR at sexual rest without sacrificing the bull. The following regression equation is suggested for indirect measurement of EGR:

$$\text{EGR} \times 10^9 = 14.2 + (1.54)\ (\text{sperm in depletion} \times 10^9)$$

In the depletion trials, significant numbers of sperm were collected after the tenth ejaculate. Thus, the first ten ejaculates contained 29.2 $\pm$ 2.8 billion sperm, as contrasted to 38.5 $\pm$ 3.5 billion for the complete depletions. The correlation between EGR and sperm collected in the first ten ejaculates was +0.81. Although highly significant, the latter correlation accounted for only 65% of the variability, whereas that based on all ejaculates collected accounted for 76%. Therefore, the more extensive depletions should be used for accurate results.

The data in Table 2 suggest a 16% increase in the sperm content of the caput epididymis associated with regular ejaculation at 6-7 $\times$ per week, as compared with SR bulls. However, Amann (4) demonstrated that ejaculation frequency and daily sperm output do not affect spermatogenesis. Therefore, the number of sperm entering and stored in the caput epididymis should be similar in both SR and 6-7 $\times$ bulls. Since gonadal reserves were larger in 6-7 $\times$ bulls than in SR bulls, the 16% difference probably represents bull variation in total EGR. The average sperm output for the five 6-7 $\times$ bulls during the 6- to 20-wk. period prior to slaughter was 6.8, 6.2, 5.2, 3.3, and 3.2 billion sperm per day and averaged 4.9 billion. Although there was an apparent decrease of 28% in the sperm content of the cauda epididymis, only 16% of the total EGR of the 6-7 $\times$ bulls was accounted for by sperm ejaculated on the day of slaughter.

Corrections for bull variation can be made either on the basis of gonadal sperm reserves (22) or the sperm content of the caput and corpus epididymis (4). The total number of sperm in the paired caput and corpus epididymis averaged 24.2 billion for SR bulls and 27.7 billion for the 6-7 $\times$ bulls. Since testis counts were not available for all bulls, the correction was made by dividing the total number of sperm in the caput and corpus epididymis into the total number contained in the cauda epididymis, ductus deferens, ampulla and ejaculated semen. The value calculated was 195% for SR bulls and 146% for 6-7 $\times$ bulls. These values suggest that there was a 25% reduction (11.8 billion

sperm) in the distal portion of the excurrent duct system of the 6-7 × bulls just prior to ejaculation, as compared with its content had these bulls been sexually rested. This difference resulted primarily from a reduction in the number of sperm in the cauda epididymis. Therefore, regular ejaculation at 6-7 × results in a marked reduction in the sperm content of the cauda epididymis even at the high point of its refilling cycle. The EGR of the 35 bulls averaged 72.6 billion sperm when corrected for variations in preslaughter treatment.

Although EGR was significantly correlated with both paired testes and paired epididymides weights (Table 3), the correlation with total sperm removed by depletion was much higher. Testis weight and epididymis weight were highly significantly correlated. In spite of heterogeneity in age and preslaughter treatment of the 25 bulls, testis sperm numbers were highly significantly correlated with testis weight and with numbers of sperm in the caput and/or corpus epididymis. However, the caput and corpus account for only 35% of the EGR of SR bulls. The correlation between testis sperm and sperm in the cauda epididymis of SR bulls was not significant (−0.16), whereas that for SR-D bulls was highly significant (+0.93). Likewise, the correlations between the sperm in the caput and in the cauda for SR bulls (+0.07) and for SR-D bulls (+0.69) suggest that much of the variability in the EGR among SR bulls was associated with the storage capacity or distensibility of the cauda epididymis. It appears that the sperm reserves in the cauda were almost entirely removed during depletion and that in the short interval between depletion and slaughter a more or less uniform percentage of the sperm in the caput and corpus moved into the essentially void cauda epididymis.

DISCUSSION

The average of 20.6 billion sperm for 50 testes was considerably less than that of 29.9 billion for 12 bull testes reported by Ortavant (21), but larger than the 8.4 billion found by Frederick (13) for testes from four 2-yr.-old bulls. These differences reflect primarily variations in technique rather than testicular weight. Based on sperm per gram of testis, values in the literature for boars (17), rams during the breeding season, rams in the nonbreeding season (22), and bulls (21) are 134, 105, 80, and 99 million, respectively, as compared to 55 million for the bulls in the present study. In rams, Ortavant (22) concluded that gonadal reserves represented spermatids in Stages II to VIII of the seminiferous epithelial cycle, whereas Amann (4) suggested that in bulls these sperm represented spermatids in Stages VI to VIII. In boars, each stem spermatogonium is reported to result in 96 spermatids, as compared to 64 in rams and bulls (4, 23). Thus, there are certain species as well as age differences which should be reflected in gonadal reserves. Nevertheless, in all three species there is a high correlation between testis weight and testis sperm (17, 22).

As discussed by Amann (4), gonadal reserves of bulls represent about 3.27 days' sperm production. Thus, it was estimated that the 25 bulls in the present study had an average daily testicular production of about 13.1 billion sperm.

The EGR of SR bulls were larger than values reported by Ortavant (23). Although he found that the paired epididymides contained 51 billion sperm, as contrasted to 62 billion in the present study, the percentage distribution of sperm in the caput, corpus, and cauda was similar to that for the SR bulls. Average epididymal reserves of 44 billion were reported by Frederiek (13) and 26 billion for slaughterhouse bulls of unknown age and sexual history by Bialy and Smith (7). The latter value, in fact, is considerably less than that for bulls under 2 yr. of age (41 billion) reported by Ortavant (23) or the SR-D bulls in Table 2 (33 billion). In sexually rested boars (17) and rams (22), the epididymal sperm reserves are 193 and 160 billion, as compared to 62 billion for SR bulls. The relative distribution of sperm in the caput, corpus, and cauda also differs among species; for rams (22), these values are 14, 7, and 79% and for SR bulls 31, 8, and 61%, respectively. These data suggest that a larger percentage of the EGR is available for ejaculation in a short period of time (depletion) in rams than in bulls. Contrary to the suggestion of Ortavant (21), this does not mean that bulls should not be collected as frequently as rams. The daily testicular sperm production of rams and bulls appears to be similar. Even with daily ejaculation, once the equilibrium level has been established in the cauda epididymis, the actual magnitude of the EGR should have little bearing on the number of sperm which can be collected (4). Daily sperm output then becomes a simple function of sperm production and sperm resorption.

Collection of bulls at one or two ejaculates per week does not affect EGR just prior to ejaculation (4). However, in the 6-7 × bulls a reduction of 25%, or 12 billion sperm, in the distal part of the excurrent duct system just prior to ejaculation was calculated as compared with the value expected had they been sexually rested. A similar reduction (less than 30%) was observed in the cauda epididymis of rams electro-ejaculated daily (22).

The value of 53% of the EGR removed by depletion from the SR-D bulls is comparable to values of 56% for rabbits calculated from the data of Edwards (12) and 40 to 45% for golden hamsters reported by Chang and Sheaffer (11), but higher than that of 31% for rams (24). In all species, sperm removed by depletion appeared to come primarily from the cauda epididymis. The reduction observed in the caput of the SR-D bulls has not been reported previously. This rapid movement of sperm from the caput and corpus into the cauda epididymis during or following depletion accounts in part for the reported (3, 13, 15, 18) inability to completely exhaust the sperm supply of bulls by repeated ejaculation.

Knowledge of the EGR is important both in the design of experiments on male reproductive physiology (1, 4) and in bull management. Thus, the highly significant correlation between EGR and sperm removed during a depletion of approximately 20 ejaculates is of great importance since it provides a convenient means of estimating EGR in living bulls.

The low correlation between body weight and paired testes weight is similar to those reported by Willett and Ohms (28) for bulls and Kennelly (17) for boars, but much lower than the value of +0.90 calculated for bulls by VanDemark (25). The highly significant correlation between testis and epididymis

weights agrees with that reported for boars (17). In both boars (17) and the SR and SR-D bulls, epididymal weight and epididymal sperm reserves were highly significantly correlated. The correlation between testis weight and epididymal sperm reserves also was significant. Thus, certain parameters reported in the literature (8, 12, 13, 25, 26, 28) to be associated with testes weight or testes sperm production actually may be associated with epididymal weight and ultimately with epididymal sperm reserves. Testis sperm and sperm in the epididymis were significantly correlated (+0.69) in 18-mo.-old sexually rested rams (22), as contrasted to the lack of correlation in the SR bulls (+0.14). Presumably, this represents an age or species difference, although it may result from technique variations.

These correlations, as well as other data (4), emphasize that sperm production and sperm storage (EGR) are not necessarily related. Thus, a single depletion of about 20 ejaculates estimates EGR but not testicular sperm production. Since several workers have attempted to estimate testicular sperm production from a series of depletion trials, the relationships among sperm production, EGR, and number of sperm removed in a depletion trial appear to merit further discussion. Based on the technique of Edwards (12), Willett and Ohms (28) calculated testicular sperm production from a series of four depletion trials (13 to 16 ejaculates) at seven-day intervals. From six 9-yr.-old bulls they obtained an average of 33.6 billion sperm per depletion, which was interpreted to represent a daily production of 6.7 million sperm per gram of testis. However, since the EGR is replenished within about seven days following a single depletion (8, 15), depletions at intervals of seven days actually represent replicate estimations of EGR.

A second approach used for measuring testicular sperm production is a regression technique involving a series of partial depletions at varying intervals between one and 13 days after an initial depletion of EGR (8, 13, 25, 26). Sperm production values of 1.95 and 1.94 billion per day have been estimated using this approach (8, 13). Considering testis weight, the daily production was reported as 2.88 million sperm per gram (8) and 1.64 million sperm per gram of testis (13). These values are much lower than the daily production of 17.5 million sperm per gram of testis based on the testis sperm counts for the 25 bulls reported herein and daily sperm output values (1, 3, 14, 15).

Certain aspects of the technique for calculating sperm production from linear regression of the total sperm collected in a depletion trial and the interval from the previous depletion trial probably account for this difference. The regression technique appears to be based on assumptions that (a) the EGR is drastically reduced in the initial depletion trial, (b) the entire testicular sperm production contributes to the EGR, and (c) the sperm removed in each depletion following the initial depletion trial represents the degree of replenishment of the EGR from a consistent low value resulting from the initial depletion. As used by various workers (8, 13, 25, 26) these assumptions, if correct, were not satisfied. Boyd and VanDemark (8) removed an average of 19.5 billion sperm in the initial depletion of their 2-yr.-old bulls, or about 40% of the EGR (4, 21).

Thus, EGR were not drastically reduced by the initial depletion. Frederick (13) obtained 29.2 billion, or 50 to 55%, of the EGR of his four young and two mature bulls. The rapid movement of sperm from the caput and corpus into the cauda epididymis, where they are available for ejaculation, as reported herein, also would confound the results. Recently, it was demonstrated that the rate of sperm resorption in the excurrent ducts is very high (4). Therefore, the entire testicular sperm production does not contribute to replenishment of the EGR. Finally, the design of the experiments resulted in a confounding of the EGR level for the depleted state from which replenishment was estimated. The replenishment was calculated from the interval between any two depletions within a series representing one replicate of a Latin square. For example, with a sequence of initial, one-, four-, and seven-day depletions, the four-day depletion represented replenishment from the double depletions of the initial and first days. A period of 15 to 30 days should be used between each pair of depletions, e.g., initial and Day One, initial and Day Four, etc. However, even with this approach and using adequate depletion trials, the values obtained would represent only the net rate of replenishment of EGR (production minus resorption)—not testicular sperm production.

The EGR of a bull at any given time is a complex function of the rates of testicular sperm production, epididymal sperm resorption, and sperm removal by collection and masturbation (4). After establishing equilibrium among these variables, the best estimate of testicular sperm production in living bulls can be obtained by collecting one or two ejaculates daily with intensive sexual preparation over an extended period of time (1, 4, 14, 18). Details of the technique for estimating testicular sperm production from daily sperm output will be presented in a forthcoming report. EGR of SR bulls can be estimated from the number of sperm removed in a suitable depletion trial.

ACKNOWLEDGMENT

The authors express their appreciation to Dr. W. T. O'Dell, Mrs. A. Keene, and Messrs. R. G. Saacke, G. R. Howe, E. W. Wickersham, R. G. Forsht, and R. F. Moyer for their technical assistance.

REFERENCES

(1) Almquist, J. O., Amann, R. P., and Hale, E. B. Measurement of Sperm Production and Its Relation to Sperm Output and Reserves in Dairy Bulls. IVth Intern. Congr. Animal Reproduction (The Hague) In press.

(2) Almquist, J. O., Amann, R. P., and O'Dell, W. T. Sperm Reserves of Dairy Bulls as Determined by Depletion Trials and Postslaughter Sperm Counts. J. Dairy Sci., 41: 733. 1958.

(3) Almquist, J. O., and Hale, E. B. An Approach to the Measurement of Sexual Behavior and Semen Production of Dairy Bulls. IIIrd Intern. Congr. Animal Reproduction (Cambridge), Plenary Papers: 50. 1956.

(4) Amann, R. P. Reproductive Physiology of the Male Bovine. Ph.D. thesis, The Pennsylvania State University, University Park. 1961.

(5) Amann, R. P., and Almquist, J. O. Reproductive Capacity of Dairy Bulls. I. Technique for Direct Measurement of Gonadal and Extra-Gonadal Sperm Reserves. J. Dairy Sci., 44: 1537. 1961.

(6) BIALY, G., AND SMITH, V. R. The Distribution of Spermatozoa in the Bovine Male Reproductive Tract. (Abstr.) J. Animal Sci., 15: 1305. 1956.

(7) BIALY, G., AND SMITH, V. R. Number of Spermatozoa in the Different Parts of the Reproductive Tract of the Bull. J. Dairy Sci., 41: 1781. 1958.

(8) BOYD, L. J., AND VANDEMARK, N. L. Spermatogenic Capacity of the Male Bovine. I. A Measurement Technique. J. Dairy Sci., 40: 689. 1957.

(9) CHANG, M. C. Disintegration of Epididymal Spermatozoa by Application of Ice to the Scrotal Testis. J. Exptl. Biol., 20: 16. 1943.

(10) CHANG, M. C. The Sperm Production of Adult Rams in Relation to Frequency of Semen Collection. J. Agr. Sci., 35: 243. 1945.

(11) CHANG, M. C., AND SHEAFFER, D. Number of Spermatozoa Ejaculated at Copulation, Transport into the Female Tract, and Present in the Male Tract of the Golden Hampster. J. Heredity, 48: 107. 1957.

(12) EDWARDS, J. The Effect of Unilateral Castration on Spermatogenesis. Proc. Roy. Soc. (London), Series B, 128: 407. 1940.

(13) FREDERICK, E. C. Sexual Performance in Dairy Bulls as Related to Frequency of Ejaculation. Ph.D. thesis, University of Minnesota, St. Paul. 1958.

(14) HAFS, H. D., HOYT, R. S., AND BRATTON, R. W. Libido, Sperm Characteristics, Sperm Output, and Fertility of Mature Dairy Bulls Ejaculated Daily or Weekly for Thirty-two Weeks. J. Dairy Sci., 42: 626. 1959.

(15) HALE, E. B., AND ALMQUIST, J. O. Relation of Sexual Behavior to Germ Cell Output in Farm Animals. J. Dairy Sci., 43 (Suppl.): 145. 1960.

(16) HALE, E. B., ALMQUIST, J. O., AND THACKER, D. L. Observations on the Sexual Behavior and Semen Production of Dairy Bulls. J. Dairy Sci., 36: 576. 1953.

(17) KENNELLY, J. J. Spermatogenesis in Boars. I. The Testicular and Epididymal Spermatozoan Reserves. II. Estimated Potential Daily Production of Spermatozoa. M.S. thesis, Cornell University, Ithaca, N. Y. 1960.

(18) KIRILLOV, V., AND MOROZOV, V. The Influence of Sexual Activity of Bulls on Their Reproductive Ability. Problemy Zhivotnovodsta, 5: 90. 1933. [Abstr., Animal Breeding Abstr., 1: 237. 1934.]

(19) MACIRONE, C., AND WALTON, A. Fecundity of Male Rabbits as Determined by "Dummy Matings." J. Agr. Sci., 28: 122. 1938.

(20) NOVOSELJCEV, D. V. O zapase zivčikov v pridatkah semmenikov hrjakov i o zakonomernostjah v rashodovani i etogo zapasa. (The Quantity of Spermatozoa in the Boar's Epididymides and Principles Governing It.) Sovet. Zootekh., 6: 76. 1951. [Abstr., Animal Breeding Abstr., 20: 58. 1952.]

(21) ORTAVANT, R. Spermatogénèse et Réserves Spermatiques chez le Taureau et le Bélier. IIIrd Intern. Congr. Animal Reproduction (Cambridge), 1: 44. 1956.

(22) ORTAVANT, R. Le Cycle Spermatogénétique chez le Bélier. D.Sc. thesis, University of Paris, Paris, France. 1958.

(23) ORTAVANT, R. Spermatogenesis and Morphology of the Spermatozoon. Reproduction in Domestic Animals, Vol. II, Chap. I. Academic Press, New York. 1959.

(24) POLOVCEVA, V. V. Skorostj prodvizenija i vremja sozrevanija spermatozoidov v epididimise barana. (The Rate of Movement and the Time of Maturation of Spermatozoa in the Epididymis of the Ram.) Doklady Akad. Sel'sko-Khoz. Nauk., 15/16: 43. 1938. [Abstr., Animal Breeding Abstr., 7: 227. 1939.]

(25) VANDEMARK, N. L. Quantitative Aspects of Semen Production in Bulls. IIIrd Intern. Congr. Animal Reproduction (Cambridge), Plenary Papers: 80. 1956.

(26) VANDEMARK, N. L., AND MAUGER, R. E. The Effect of Energy Intake and Growth on the Spermatogenic Capacity of Young Bulls. (Abstr.) J. Animal Sci., 16: 1042. 1957.

(27) WALTON, A., AND EDWARDS, J. Criteria of Fertility in the Bull. I. The Exhaustion Test. Proc. Am. Soc. Animal Production, 31: 254. 1938.

(28) WILLETT, E. L., AND OHMS, J. I. Measurement of Testicular Size and Its Relation to Production of Spermatozoa by Bulls. J. Dairy Sci., 40: 1559. 1957.

ACID-SOLUBLE PHOSPHORUS CONSTITUENTS OF BULL SEMEN IN RELATION TO FRUCTOLYSIS AND TO SPERM CONCENTRATION[1, 2]

M. H. EHLERS, JOAN DIXON, AND R. E. ERB
Department of Dairy Science, Washington State University, Pullman

SUMMARY

Eighty-seven semen samples from five bulls had an average phosphorus content (P_{Tot}) of 0.807 mg. per milliliter and these acid-soluble phosphorus values in milligrams per milliliter: 0.039 inorganic (P_I), 0.046 inorganic + easily hydrolyzable (P_{I+H}), and 0.460 total acid-soluble (P_{TAS}). During 3 hr. of incubation of these samples in citrate buffer, P_I increased 0.013 ($P < 0.01$); P_{I+H} increased 0.008 ($P < 0.01$); but P_{TAS} did not significantly change. Increase in P_I was positively correlated with sperm concentration, but negatively correlated with fructose utilization. Thirty-two of these samples, based on hourly incubation data, so depleted their fructose content during the second or third hour (< 0.25 mg. per milliliter incubate remaining) that utilization was halved, compared with the hour prior to this depletion. P_I, however, showed a similar increase during both hours of comparison. Fifteen samples adjusted with seminal plasma to ½, 1, and 2 × the original sperm concentration indicated that during 3 hr. of incubation increase in P_I was largely, but not wholly, a function of sperm numbers, where sperm were from a similar source. The data do not give conclusive evidence as to the source of increase in P_I in bull semen, but they do not contradict the concept that the increase is partially due to irreversible breakdown of ATP. Coupled with a report of P_I increase upon cold shock of ram sperm, it is suggested that the increase indicates degenerative or senescent change.

Within an hour of ejaculation, human semen shows a rapid increase in inorganic phosphorus (P_I), due to the breakdown of phosphorylcholine (10). In bull semen, stable glycerylphosphorylcholine accounts for practically all of the bound choline, and acid phosphatase activity is negligible; there is little if any increase in P_I upon incubation of bull seminal plasma (3). Flerchinger and Erb (7), however, noted a small but significant increase of 8.2% in P_I upon the incubation of bull semen in citrate buffer for 1 hr. at 37° C. The data of Dawson *et al.* (3) can be interpreted as showing an increase in P_I with 37° C. incubation of ram semen, particularly under aerobic conditions. Mann and Lutwak-Mann (13) noted that rapid cooling of ram sperm markedly increased P_I, owing to the breakdown of ATP. Blackshaw and Salisbury (1) found that cold shock of undiluted bull semen resulted in the loss of lipid phosphorus from the sperm cell. Mann and Lutwak-Mann (13) held that leakage of cytochrome c following cold shock was an after-effect of ATP disintegration. Loss of cytochrome c from the sperm cell has been noted with the use of certain detergents (8), and has been associated with sperm senescence (11). It is thus possible

Received for publication December 1, 1960.

[1] Scientific Paper No. 2042, Washington Agricultural Experiment Stations, Pullman. Station Project 1344.

[2] This research was financed in part under Regional Research and Marketing Act, Section 963, Public Law 733. Project W-49.

that P_I increase in bull semen upon incubation is a cellular phenomenon and indicative of senescent changes.

The present study, by using more semen than could be spared in the fertility trial of Flerchinger and Erb (7), was designed to establish whether increase in P_I could be considered characteristic of all bull semen. By prolonging the incubation period and observing the effects of natural and artificial variation in sperm concentration, it was expected that any association between phosphorus change and sperm concentration, fructolytic activity, or fructose depletion, might be detected. The quantity of semen also permitted determinations of other acid-soluble phosphorus forms.

MATERIALS AND METHODS

Semen diluted with four parts of 2.9% sodium citrate dihydrate was incubated in culture tubes for 3 hr. at 37° C. At the beginning and end of controlled incubation, and at hourly intervals except for the trials involving artificial variation in sperm concentration, 2 ml. of semen-citrate were removed by pipette and forcibly discharged into 8 ml. of cold 10% trichloroacetic acid. After centrifugation and filtration, the equivalent of 0.01 ml. semen from each filtrate was used for fructose determination (5, 14).

The basic phosphorus method was that of Fiske and Subbarow (6). Phosphate in the trichloroacetic acid filtrate reacting directly with molybdate, expressed as phosphorus, was termed P_I, or inorganic phosphorus. That which reacted with molybdate after 7 min. acid hydrolysis was termed $P_{I,H}$, in recognition that this included inorganic and easily hydrolyzable phosphate. That which reacted after wet-ashing the filtrate was termed P_{TAS}, or total acid-soluble phosphorus. Total phosphorus, P_{Tot}, was determined by wet-ashing the sample without subjecting it to prior precipitation with trichloroacetic acid. For P_I, $P_{I,H}$, and P_{TAS}, the equivalent of 0.2, 0.08, and 0.016 ml. semen in trichloroacetic acid filtrate was used; the equivalent of 0.016 ml. semen was used for P_{Tot} determination. In the acid hydrolysis procedure, tubes containing equal parts (2 ml.) of filtrate and *N* hydrochloric acid were placed in a boiling water bath for 7 min. For wet-ashing to obtain P_{TAS} and P_{Tot}, tubes containing 2 ml. of a solution having the desired semen equivalent and 0.5 ml. of concentrated sulfuric acid, with beads to minimize bumping, were heated 45 min. over an electric unit and again heated 15 min. after the addition of 0.5 ml. of 70% perchloric acid. Subsequent to these various treatments, phosphorus determinations were made, using appropriate molybdate solutions (6).

In trials involving artificial adjustment of sperm concentration, samples were centrifuged 10 min., a portion of the plasma removed, the sperm resuspended, and plasma recombined to ½, 1, and 2 × the original sperm concentration. A limited number of sperm were retained in the plasma, even with repeated centrifugation, and the sperm concentration actually recorded was adjusted by sperm counts of the plasma, using a hemocytometer. Sperm counts for other work were estimated photometrically. Correlation analyses and tests for significance were according to Snedecor (15).

TABLE 1

Phosphorus composition, sperm concentration, sperm motility, and fructolytic data on 87 samples of bull semen from five bulls incubated 3 hr. at 37° C. in four parts sodium citrate buffer

Measurement		Mean	Std. dev.	95% confidence limit [a]	Previous reports (references in parentheses) [a]
		(mg. per ml. semen)			
P_{Tot} or total phosphorus (phosphate reaction with molybdate after wet-ashing)		0.807	0.408	0.721 to 0.893	0.82 mg. in semen of 0.985×10^9 sperm (12) 1.459 mg. in semen of 1.672×10^9 sperm (7) 1.02 mg. in semen of unspecified $\times 10^9$ sperm (2) 0.78-1.32 mg. confidence interval calculated
Acid-soluble phosphorus (in trichloracetic acid filtrate)					
P_I or inorganic phosphorus (phosphate reacting directly with molybdate)	0 hr.	0.039	0.025	0.034 to 0.044	0 hr. = 0.170 mg. (7); 0.09 mg. (12); 0.012 mg. (9)
	3 hr.	0.052	0.029	0.046 to 0.058	
	Change [b]	0.013	0.014	0.010 to 0.016	
P_{I+H} (phosphate reacting with molybdate after 7 min. acid hydrolysis; includes inorganic)	0 hr.	0.046	0.024	0.041 to 0.052	
	3 hr.	0.054	0.027	0.048 to 0.059	
	Change [b]	0.008	0.008	0.006 to 0.010	
P_{TAS} or total acid soluble phosphorus (phosphate reacting with molybdate after wet-ashing; includes easily hydrolyzable).	0 hr.	0.460	0.168	0.424 to 0.496	0 hr. = 0.33 mg. (12); 0.170 mg. typical (9)
	3 hr.	0.470	0.205	0.428 to 0.515	
	Change	0.010	0.115	0.015 to 0.036	
Fructose	0 hr.	5.20	1.03		
	3 hr.	1.36	1.31		
	Change [b]	3.84	1.08		
		$\times 10^9$/ml. semen			
Sperm concentration		1.02	0.38		
		Per cent progressively motile			
Sperm motility	0 hr.	67	8		
	3 hr.	23	18		

[a] Phosphorus data only.
[b] Significant change ($P < 0.01$) during 3 hr. of incubation.

RESULTS AND DISCUSSION

From various experiments, 87 semen samples (five bulls) had 0- and 3-hr. incubation values for percentage progressive sperm motility, fructose, and the three measurements of acid-soluble phosphorus (P_I, P_{I+H}, P_{TAS}). Average values for these, together with P_{Tot} and sperm concentration, are indicated in Table 1. Conventional rates of fructose utilization adjusted for sperm concentration were not calculated, because the intentionally long incubation, with subsequent near-exhaustion of fructose in many samples, made such rate calculation invalid.

The other studies summarized in Table 1, together with the present findings, appear to agree as to P_{Tot} in bull semen, particularly when attention is given to variation in sperm concentration. It is thus believed that lack of agreement as to the amounts of different phosphorus fractions is due more to variation in laboratory procedures than to semen sources.

Although present values for P_I are less than noted my Flerchinger and Erb (7), the 33% increase upon 3 hr. of incubation is comparable with the 8.2% increase noted by them in the shorter (1-hr.) incubation.

As noted in Table 1, P_I increased 0.013 mg. in 3 hr. ($P > 0.01$) and P_{I+H} increased 0.008 mg. ($P > 0.01$). If P_{I+H} had remained unchanged, the increase in P_I might have been attributed wholly to the breakdown of pyrophosphates. The present results suggest that part of the increase in P_I came from other sources, since P_{I+H} showed some increase with incubation. P_{TAS} in the present study did not alter significantly upon incubation at 37° C. Lardy and Phillips (9) noted an increase to 0.236 mg. from an initial 0.170 during 56 hr. of semen storage at 10° C.

Table 2 presents correlations between the three acid-soluble phosphorus measures and sperm concentrations, and between these measures and frucolytic data. The sample within bull correlations are probably most informative and subsequent comments will refer to them. Positive correlations between sperm concentration and the three 0-hr. acid-soluble phosphorus measures, particularly P_{TAS}, merely reflect the general association of semen phosphorus with the sperm cell. The correlation ($r = 0.39$) between sperm concentration and P_I increase supports the concept that the increase is a function of cell metabolism. Flerchinger and Erb (7) failed to note a relationship between sperm concentration and increase in P_I ($r = -0.05$), probably because these small changes are more difficult to detect in a shorter incubation, and because lesser quantities of semen were used in their analyses.

The generally negative nature of correlation between 0-hr. fructose and the three acid-soluble phosphorus measures partially reflect the commonly noted negative relation between sperm concentration and seminal fructose, as may be observed in the partial correlations independent of sperm concentration (Table 2). The generally smaller magnitude of correlations between 3-hr. fructose and acid-soluble phosphorus measures, none of which is significant in the partial correlations independent of sperm concentration, is probably due to the near depletion of fructose in many samples (Table 2).

TABLE 2

Correlations between three acid-soluble phosphorus measures and sperm concentration and fructolytic measures on 87 samples of bull semen from five bulls

Comparison		Total r	Among bulls r	Sample within bull r	Sample within bull $r_{12.3}$
Degrees of freedom $(n-2)$, $(n-3)$		85	3	82	81
Sperm concentration × acid-soluble phosphorus measures					
P_I	0 hr.	+.21[b]	+.37	+.23[b]	.....
	3 hr.	+.39[a]	+.65	+.35[a]	.....
	Change	+.47[a]	+.72	+.39[a]	.....
P_{I+II}	0 hr.	+.22[b]	−.13	+.30[a]	.....
	3 hr.	+.30[a]	+.28	+.33[a]	.....
	Change	+.28[a]	+.78	+.19	.....
P_{TAS}	0 hr.	+.64[a]	+.81	+.62[a]	.....
	3 hr.	+.58[a]	+.93[b]	+.53[a]	.....
	Change	+.05	+.40	−.02	.....
Fructose 0 hr. × acid-soluble phosphorus measures					
P_I	0 hr.	−.33[a]	−.41	−.43[a]	−.39[a]
	3 hr.	−.50[a]	−.78	−.51[a]	−.42[a]
	Change	−.51[a]	−.87[b]	−.35[a]	−.21[b]
P_{I+II}	0 hr.	−.21[b]	+.16	−.34[a]	−.24[b]
	3 hr.	−.32[a]	−.39	−.39[a]	−.27[b]
	Change	−.37[a]	−.61	−.26[b]	−.20
P_{TAS}	0 hr.	−.42[a]	−.59	−.42[a]	−.19
	3 hr.	−.36[a]	−.71	−.30[a]	−.07
	Change	−.01	−.34	+.07	+.02
Fructose 3 hr. × acid-soluble phosphorus measures					
P_I	0 hr.	−.17	−.35	−.19	−.08
	3 hr.	−.24[b]	−.88[b]	−.12	+.04
	Change	−.22[b]	−.39	−.17	+.04
P_{I+II}	0 hr.	−.16	+.20	−.23[b]	−.09
	3 hr.	−.18	−.27	−.19	−.03
	Change	−.13	−.93[b]	+.02	+.14
P_{TAS}	0 hr.	−.46[a]	−.72	−.42[a]	−.15
	3 hr.	−.43[a]	−.86	−.35[a]	−.11
	Change	−.08	−.41	−.02	−.01
Fructose utilization × acid-soluble phosphorus measures					
P_I	0 hr.	−.24[b]	−.44	−.24[b]	−.25[b]
	3 hr.	−.38[a]	−.79	−.33[a]	−.35[a]
	Change	−.40	−.90[b]	−.31	−.35[a]
P_{I+II}	0 hr.	−.16	+.09	−.18	−.19
	3 hr.	−.20	−.40	−.19	−.20
	Change	−.34[a]	−.93[b]	−.28[b]	−.29[b]
P_{TAS}	0 hr.	−.01	−.17	+.01	0
	3 hr.	−.02	−.23	+.01	0
	Change	+.06	−.11	+.08	+.08

r—Simple correlation.
$r_{12.3}$—Partial correlation independent of sperm concentration.
[a] Correlation $P < 0.01$.
[b] Correlation $P < 0.05$.

TABLE 3

P_I and P_{I+II} increase as related to adequacy of fructose to support metabolism during a 3-hr. incubation at 37° C.

Fructose adequacy	n	Sperm concentration $\times 10^9$/ml semen	Mg. phosphorus per ml. semen						Mg. fructose per ml. semen		
			P_I			P_{I+II}					
			0 hr.	3 hr.	Change	0 hr.	3 hr.	Change	0 hr.	3 hr.	Utilization
Semen having $\geqq 0.25$ mg/ml incubate at end of 3 hr. ($\geqq 1.25$/ml semen)	36	0.74	0.035	0.042	0.007	0.043	0.048	0.005	6.25	2.52	3.73
Semen having < 0.25 mg/ml incubate at end of 3 hr.	51	1.21	0.040	0.057	0.017	0.049	0.057	0.009	4.45	0.54	3.90

TABLE 5

P_I and P_{I+II} in 15 semen samples adjusted to 2 ×, 1 ×, and ½ × original sperm concentration and incubated 3 hr. at 37° C.

Treatment	Actual sperm[a] concentration per ml. semen $\times 10^9$	Sperm motility (%)		Phosphorus mg. per ml. semen						Fructose mg. per ml. semen		
					P_I			P_{I+II}				
		0 hr.	3 hr.	0 hr.	3 hr.	Change	0 hr.	3 hr.	Change	0 hr.	3 hr.	Utilization
2 ×	2.20	68	18	0.048	0.072	0.024	0.070	0.088	0.012	4.46	0.90	3.56
1 ×	1.14	67	26	0.042	0.058	0.016	0.060	0.069	0.009	5.02	1.55	3.47
½ ×	0.64	67	36	0.039	0.047	0.008	0.054	0.060	0.006	5.45	2.61	2.84

[a] Adjusted for sperm which failed to separate from plasma during centrifugation.

Fructose utilization × 0 hr., 3 hr., and increase in P_I were negatively correlated (Table 2). The latter correlation ($r = -0.31$, partial independent of sperm concentration $= -0.35$) is intriguing, because of its metabolic implications. This suggests that with lowered fructose utilization, probably associated with lack of available substrate rather than defective sperm, there is greater P_I increase.

The 87 samples were divided into two categories (Table 3), dependent upon the adequacy of available fructose to support normal fructolytic activity for 3 hr. at 37° C. Samples having 0.25 mg. or more of fructose per milliliter incubate at the end of 3 hr. (1.25 mg/ml semen) were considered to have had adequate fructose throughout the incubation; whereas, those with less than 0.25 mg. per milliliter of incubate were regarded not adequate (4). The data of Table 3 support the position that increase in P_I is positively associated with sperm concentration or fructose depletion or both, but not with fructose utilization, since utilization on a per-milliliter semen basis was similar in both categories.

In a further investigation of the relationship between fructolytic activity and P_I increase, 72 samples having hourly incubation determinations of phosphorus and fructose were examined. Thirty-two of these samples had depleted their fructose during the second or third hour of incubation, so that less than 0.25 mg. per milliliter incubate (1.25 mg. per milliliter semen) remained. This hour, during which the level of fructose became critical (4), was compared with the previous hour of incubation, as shown in Table 4. Results indicate that P_I and P_{I+H} continued to increase, even though fructose utilization dropped to about one-half the level of that during the prior hour, when the fructose level was not a limiting factor.

Table 5 presents data on 15 samples artificially adjusted to ½, 1, and 2 × the original sperm concentration and similarly incubated for 3 hr. The ½ × portions showed one-half the increase in P_I of the 1 ×; the 2 × portions had less than twice the increase of the 1 ×, indicating that the increase in P_I is largely, but not wholly, a function of sperm numbers when sperm are derived from a common source. Although P_{I+H} increased with higher sperm numbers, the increase was apparently not so pronounced as with P_I. This might mean more ATP breakdown in samples with higher sperm concentration, where fructose

TABLE 4

Comparison of fructose and phosphorus changes per milliliter semen preceding and during hour in which fructose inadequacy occurred [a] ($n = 32$)

Description	Fructose	P_I	P_{I+H}
		(mg.)	
Quantity at beginning of hour during which fructose became inadequate	1.91	0.043	0.047
Fructose utilization or phosphorus increase during preceding hour of incubation	1.86	0.004	0.001
Fructose utilization or phosphorus increase during hour at which fructose became inadequate	0.90	0.005	0.003

[a] < 0.25 mg. per milliliter incubate.

was inadequate to support normal fructolysis. Sperm motility was similar in the treatments initially, but at 3 hr. was inversely related to sperm concentration in reflection of the fructose depletion (4). The lesser increase in P_I in the 2 × portions, when sperm numbers are considered, may have been associated with the lesser motility at 3 hr., or with an earlier decline in motility.

GENERAL DISCUSSION

The positive correlation between sperm concentration and increase in P_I upon incubation, the negative correlation between fructose utilization and increase in P_I, the comparisons of successive hourly incubations upon P_I and fructose as the latter becomes inadequate, and the results with artificially adjusting sperm concentration indicate that inorganic phosphorus increase in incubating bull semen is (a) a function of cellular metabolism but (b) not merely indicative of over-all fructolytic acitvity.

The negative correlation between P_I increase and fructose utilization, particularly the partial correlation independent of sperm concentration, coupled with the observation on ram semen of increase in P_I with cold shock (13), suggests that this increase is indicative of deterioration.

It may be noted, however, that P_I increase in the samples with artificially high sperm concentration was not as great as would be expected if fructose depletion accelerated the P_I increase.

Although the present data are not conclusive, the lesser increase in easily hydrolyzable phosphorus upon incubation of semen does not contradict the concept that ATP breakdown is at least partially the source of the increased P_I. The relative constancy of P_{TAS} indicates that little, if any, phosphorus is changing from a lipid or nucleic acid designation to an acid-soluble form under these conditions of 37° C. incubation.

The apparent usefulness of these observations is that they suggest the kind of changes which may occur in sperm with fructose utilization and depletion. Furthermore, even though fructose is present in adequate quantities, P_I does increase. It would appear desirable to establish conditions which accelerate or decelerate this P_I accumulation. Finding the source of increase in P_I will probably require techniques different from those used in these experiments.

REFERENCES

(1) Blackshaw, A. W., and Salisbury, G. W. Factors Influencing Metabolic Activity of Bull Spermatozoa. II. Cold Shock and Its Prevention. J. Dairy Sci., 40: 1099. 1957.

(2) Cragle, R. G., Salisbury, G. W., and Muntz, J. H. Distribution of Bulk and Trace Minerals in Bull Reproductive Tract Fluids and Semen. J. Dairy Sci., 41: 1273. 1958.

(3) Dawson, R. M. C., Mann, T., and White, I. G. Glycerylphosphorylcholine and Phosphorylcholine in Semen and Their Relation to Choline. Biochem. J., 65: 627. 1957.

(4) Erb, R. E., Albright, J. L., and Ehlers, M. H. Influence of Prolonged Incubation of Bull Semen at 37° C. on Fructolysis, Lactic Acid, and Motility. J. Dairy Sci., 42: 1216. 1959.

(5) Erb, R. E., Flerchinger, F. H., Ehlers, M. H., and Gassner, F. X. Metabolism of Bull Semen. II. Fructolysis Relationships with Sperm Concentration and Fertility. J. Dairy Sci., 39: 326. 1956.

(6) FISKE, C. H., AND SUBBAROW, Y. The Colorimetric Determination of Phosphorus. J. Biol. Chem., 66: 375. 1925.

(7) FLERCHINGER, F. H., AND ERB, R. E. Metabolism of Bull Semen. I. Inorganic and Total Phosphorus Relationships. J. Dairy Sci., 38: 1028. 1955.

(8) KOEFOED-JOHNSEN, H. H., AND MANN, T. Studies on the Metabolism of Semen 9. Effect of Surface Active Agents with Special Reference to the Oxidation of Succinate by Spermatozoa. Biochem. J., 57: 406. 1954.

(9) LARDY, H. A., AND PHILLIPS, P. H. The Interrelation of Oxidative and Glycolytic Processes as Sources of Energy for Bull Spermatozoa. Am. J. Physiol., 133: 602. 1941.

(10) LUNDQUIST, F. Function of Prostatic Phosphatase. Nature, 158: 710. 1946.

(11) MANN, T. Studies on the Metabolism of Semen 7. Cytochrome in Human Spermatozoa. Biochem. J., 48: 386. 1951.

(12) MANN, T. The Biochemistry of Semen. Methuen and Co., Ltd., London. 1954.

(13) MANN, T., AND LUTWAK-MANN, C. Biochemical Changes Underlying the Phenomenon of Cold Shock in Spermatozoa. Arch. Sci. Biol., 39: 578. 1955.

(14) ROE, J. H. A Colorimetric Method for the Determination of Fructose in Blood and Urine. J. Biol. Chem., 107: 15. 1934.

(15) SNEDECOR, G. W. Statistical Methods. 5th ed. Iowa State College Press, Ames. 1956.

SOURCE OF ENDOGENOUS PYRUVIC ACID IN BOVINE SEMINAL FLUID AND UTILIZATION

WILLIAM G. R. MARDEN
Marden Laboratories, Bryan, Texas

SUMMARY

A significantly high concentration of pyruvic acid has been found in bovine seminal vesicular fluid with concentrations of from 17 to 47 mg/ml. This endogenous pyruvic acid is very rapidly removed from the seminal plasma, following ejaculation, generally falling to less than 1 mg/ml within 1 hr. after ejaculation. This, however, is dependent upon the activity of the spermatozoa. It is concluded that this seminal pyruvic acid, like fructose, is a source of energy for the spermatozoa. Small decreases in pyruvic acid are obtained by the incubation of seminal vesicular fluid alone but, for its rapid and almost complete removal, active spermatozoa appear necessary because lyophilized whole semen loses this ability. The incubation of bovine seminal vesicular fluid with TPN (triphosphopyridine nucleotide) increased pyruvic acid in the fluid, whereas incubation alone decreased it. There is no correlation between the fructose and the pyruvic acid concentrations in bovine seminal vesicular fluid. The concentration of pyruvic acid in bovine ampullar fluid is from 4.5 to 16 mg/ml.

Spermatozoa derive their energy chiefly from two processes: namely, glycolysis and through the use of molecular oxygen. It has been well established that in the absence of oxygen, spermatozoa rely on carbohydrate metabolism as their main source of energy. This was first demonstrated by Redenz (9), who found that dialyzed spermatozoa under nitrogen became immotile. He also found that as the concentration of carbohydrate decreased that of lactic acid increased. Later, Mann (7) proved that the major carbohydrate component of seminal fluid was fructose; not glucose, as had been previously assumed. While other compounds have been identified in mammalian seminal plasma with the exception of fructose, none has been shown to act as a significant source of energy which the spermatozoa could utilize immediately following ejaculation. A high concentration of citric acid has been found in the seminal plasma of most mammals, including the bull, but it does not appear to influence markedly the aerobic, or anaerobic metabolism of spermatozoa, Humphrey and Mann (5). While demonstrating the presence of five free amino acids in bovine seminal plasma, Gassner and Hopwood (4) were unable to show the utilization of any of these after incubating whole semen for as long as 3 hr.

While investigating Keto acids in bovine seminal plasma it became apparent that endogenous pyruvic acid was present in appreciable quantities, but was utilized by motile spermatozoa so rapidly that only 30 min. after ejaculation it had practically all disappeared.

It has been shown by Lardy and Phillips (6) and by Melrose and Terner (8) that bovine spermatozoa are capable of metabolizing pyruvic acid. Though this ability was well established, the identification of pyruvic acid as a normal constituent of seminal plasma was not demonstrated.

Received for publication May 9, 1960.

EXPERIMENTAL PROCEDURES

1. *Detection of pyruvic acid as a 2,4-dinitrophenylhydrazone derivative.* A 1- or 2-ml. sample of the fluid to be investigated was made slightly acid with a drop of HCl and deproteinized by the addition of 6 vol. of 95% ethanol. After removal of the proteins by centrifugation, 1 ml. of saturated 2,4-dinitrophenylhydrazine (hereafter referred to as DNPH) in 2 *N* HCl was added to the ethanolic extract and the mixture allowed to react at room temperature for several hours. Most of the ethanol was evaporated by the use of a warm water bath under a stream of nitrogen. The remaining solution was made basic by the addition of 12 ml. of 1 *M* $NaHCO_3$ and any unreacted DNPH removed by three 12-ml. extractions with chloroform. The combined chloroform extracts were backwashed with 12 ml. of 1 *M* $NaHCO_3$ solution and the washings combined with the original carbonate layer. The combined carbonate extracts were acidified with HCl and the yellow hydrazones removed by 12-ml. extractions with ethyl acetate. The ethyl acetate was evaporated to a volume of 1 ml., at which point the extract was ready for chromatography.

2. *Chromatography of 2,4-dinitrophenylhydrazones.* Large sheets of Whatman No. 1 filter paper (18 by 22½ in.) were employed. These can accommodate up to 20 separate spot applications on a single chromatogram. The extracts were applied in two lambda increments, allowing drying between applications. A total application from two to 40 lambda was applied to each spot, depending on the concentration of the extract. Each extract was generally run in quadruplicate. In addition, a pyruvic acid hydrazone standard curve was applied on each paper. The chromatograms were run by the ascending method in a mixture of *n*-butanol, ethanol, and 0.5 *N* NH_4OH (80:5:15) for about 18 hr., after which they were dried in air and sprayed with 2.5% ethanolic KOH. The DNPH derivatives were seen as yellow or brown spots on a white background.

Quantitative measurements, using the peak density method and employing a Welch Densichron, were made on any spots appearing as single entities. Spots of known composition were measured quantitatively, employing a standard curve. These standard curves were run alongside the unknown and a new standard curve was prepared as part of each chromatogram. By this technique, less than 0.2 mg. calculated as pyruvic acid but chromatographed in the form of DNPH derivative could be detected.

3. *Identification of hydrazine derivatives.* The use of both one- and two-dimensional chromatography, using a number of solvent systems, was not deemed sufficient to identify the unknowns. To greatly strengthen the identification of any hydrazine derivative obtained from seminal secretions, a second method was employed. The method used was described by Towers (13) and consisted of converting the hydrazine derivatives of keto acids to the corresponding amino acids. This was accomplished by subjecting the derivative to an atmosphere of hydrogen under pressure in the presence of finely divided platinum. The hydrazone was split at the nitrogen-nitrogen bond, forming an amino acid which was identified by chromatography (Figure 1).

To separate the final hydrazone extract into its components, the extract was applied as a streak at the origin of a chromatogram and run as previously described.

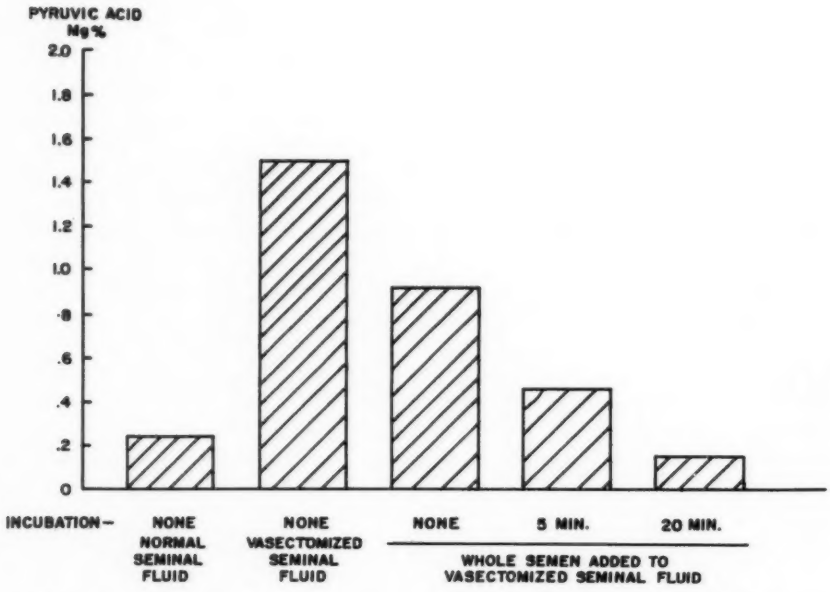
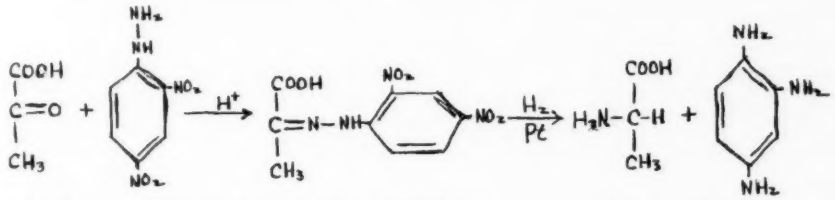


FIG. 1. Concentration and metabolism of pyruvic acid in bovine seminal fluid.

Most of the hydrazones separated into definite bands of color; these were cut out and the hydrazone eluted from the paper with warm 1 *N* $NaHCO_3$ (three extractions being sufficient). The combined carbonate extracts were cooled in the refrigerator, acidified with 6 *N* HCl, and the hydrazone extracted with two extractions of ethyl acetate. The ethyl acetate extract was then reduced in volume by evaporation, transferred to a conical centrifuge tube, and evaporated to dryness. One milliliter of distilled water was added and the tube heated until all the derivative went into solution. Several milligrams of platinum oxide were added and the centrifuge tube placed in an empty citrate bottle. A pressure of 50 lb. per square inch of hydrogen was applied for 18 to 24 hr. The centrifuge tube containing the clear aqueous solution was removed from the citrate bottle, placed in a hot water bath, and evaporated to approximately 0.2 ml., at which point the sample was ready for chromatography. The amino acid formed by the molecular splitting of the hydrazone was identified by amino acid chromatography, using both one- and two-dimensional chromatograms. These were run by the ascending method in phenol water (80:20) then in the second dimension in 2,4-lutidine, dried and sprayed with ninhydrin.

The conversion of pyruvic acid to alanine as used in proving the presence of pyruvic acid in bovine and human semen.



Pyruvic Acid | 2,4-dinitrophenylhydrazine | Pyruvic 2,4-dinitrophenylhydrazone | Amino Acid *Alanine*

RESULTS

1. *Efforts to detect pyruvic acid in bovine seminal plasma.* At the start of this investigation, it was anticipated that pyruvic acid would be readily identified in bovine seminal plasma. This proved not to be the case. Indeed, a yellow spot was obtained, which had an R_f approximately the same as one of the two isomers obtained on chromatographing the pyruvic acid hydrazone synthesized in the laboratory. However, this unknown failed to turn brown on being sprayed with 2.5% ethanolic potassium hydroxide; a characteristic of the more polar isomer of pyruvic acid hydrazone. Further, on hydrogenation the spot suspected of being derived from pyruvic acid did not give the amino acid alanine, thus proving that this spot was not a derivative of pyruvic acid. On the other hand, when the reference sample of pyruvic acid hydrazone was chromatographed and either of the two isomers obtained, eluted from the paper, and hydrogenated, only a single amino acid alanine could be obtained. It was, therefore, concluded that the unknown spot was not the hydrazone of pyruvic acid.

To obtain additional evidence, the unknown was eluted from several preparatory chromatograms where the final ethyl acetate extract had been streaked across the origin and run as previously described. The unknown band of color was cut out and eluted from the paper, crystallized twice from acetic acid, and a melting point determination made. This unknown melted at 204-206° C., while the laboratory-synthesized pyruvic acid hydrazone melted sharply at 218° C. A mixture melting point determination was made with the mixed samples melting at 181° C. Thus, a 37° C. melting point depression was obtained over that of the laboratory-synthesized pyruvic acid hydrazone.

2. *Recovery of pyruvic acid added to the bovine seminal plasma.* It was evident that we were not detecting pyruvic acid in bovine seminal plasma and, to check our method, 1,000, 500, and 250 mg. of pyruvic acid in the form of sodium pyruvate were added to 5-ml. samples of bovine seminal plasma. After following the extraction procedures already outlined, the final extracts were chromatographed along with a graded series of pyruvic acid hydrazone standards used to prepare a standard curve. The peak densities obtained from quadruple spots from each sample were averaged and the percentage recovery of pyruvic acid calculated as shown in Table 1.

TABLE 1

Recovery of pyruvate added to 5-ml. samples of bovine seminal plasma

Pyruvic acid added	Recovery
(μg.)	*(%)*
1,000	100
500	90
250	67

3. *Investigation into the seminal secretions of a vasectomized bull.* Due to our inability to prove the presence of endogenous pyruvic acid in bovine seminal plasma, our attention was turned to a vasectomized bull. This animal had been vasectomized a number of years previously, and was known to be completely bilaterally vasectomized. In a number of experiments, fluid from this bull was deproteinized and treated by the method already outlined. The final extract was chromatographed alongside a graded series of pyruvic acid hydrazone standards. On spraying the finished chromatogram with 2.5% ethanolic KOH, heavy brown spots were observed, having exactly the same R_f as our pyruvic acid standards. Due to the high concentration of this unknown hydrazone in the seminal fluid of the vasectomized bull, the possibility of making its identification even with small amounts was practical. New chromatograms were prepared by streaking the final extracts across the origin. These were chromatographed and the unknown cut out, eluted from the paper, and subjected to hydrogenation. On rechromatographing this hydrogenated material, pronounced spots of alanine were observed. Thus, it became apparent that though we had not been able to prove the presence of endogenous pyruvic acid in the bovine seminal plasma, its presence was readily detectable in the seminal fluid obtained from the vasectomized bull. Consequently, we assumed that the very faint brown streak R_f .41 previously detected in the seminal plasma of normal ejaculates, was most likely pyruvic acid, but present at such a low concentration that it had not been possible to make its positive identification.

4. *Incubation of seminal fluid of a vasectomized bull with bovine seminal plasma.* As our previous results showed that endogenous pyruvic acid was many times higher in concentration in the seminal fluid of a vasectomized bull, it was thought possible that an enzyme might be contributed by the epididymis, at the moment of ejaculation, which would trigger the utilization of pyruvic acid. To test this hypothesis, the seminal fluid from a vasectomized bull was incubated at 37° C. for 30 min. with:

(1) Bovine seminal plasma
(2) Bovine semen

The pyruvic acid content of each sample was measured quantitatively, using the peak density method already outlined.

Our results showed that a slight but definite decrease in the concentration of pyruvic acid was obtained when the seminal fluid from a vasectomized bull was incubated with an equal amount of bovine seminal plasma. On the other

hand, if the seminal fluid from a vasectomized bull was incubated with whole semen, the pyruvic acid content decreased rapidly to almost zero.

5. *Rate of utilization of pyruvic acid from vasectomized bovine seminal fluid.* To obtain some idea of the rate of utilization of pyruvic acid by whole bovine semen, it was decided to mix the seminal fluid of a vasectomized bull with one-half volume of whole semen and to incubate it at 37° C. for varying lengths of time: (zero time–immediate deproteinization; 5 and 20 min., respectively). The pyruvic acid content of each sample was measured quantitatively.

There was a marked decrease in the pyruvic acid content on mixing the vasectomized seminal fluid and whole semen, even though the mixture was deproteinized rapidly after being mixed. The pyruvic acid content dropped from 1.5 mg.% in the control to 0.92 mg.%, following addition of whole semen. After 5 min. of incubation, the level had fallen to .47 mg.% and, after 20 min., to .16 mg.%, Figure 1.

6. *Source and concentration of pyruvic acid in bovine seminal fluid.* Since our previous experiments indicated that pyruvic acid was present in the seminal fluid of the vasectomized bull and was rapidly utilized by active spermatozoa, we decided to investigate the site of production and storage of this naturally occurring seminal substrate.

The seminal vesicles and ampulla glands of seven bulls were obtained. The organs were removed within 20 min. after the animals were slaughtered and their secretions were squeezed into vials and frozen in dry ice.

The fructose and pyruvic acid content of the seminal vesicular fluid from each bull was measured quantitatively, the method of fructose determination being a modification of Roe (10), and compared with the pyruvic acid content of the ampullar fluid. These results are summarized in Table 2.

These results show clearly that bovine seminal vesicular fluid contains a high concentration of pyruvic acid, when compared with the mere trace we found so hard to identify in whole semen. (In our experiments there was always about a half-hour to 1 hr. time-lapse between the collection of whole semen and

TABLE 2

Fructose and pyruvic acid content of bovine seminal vesicular fluid and ampullar fluid

Fructose and pyruvic acid content of bovine seminal vesicular fluid				Pyruvic Acid content of ampullar fluid
Bull	Vol. obtained	Fructose	Pyruvic Acid	
	(ml.)		*(mg. %)*	
1	6	976	30	4.5
2	10	836	38	16.0
3	15	752	46	7.1
4	8	679	36	11.7
5	15	763	28.8	11.6
6	10	660	32.3	11.1
7	70	995	30	4.5
	Average	809	34.4	9.5

its delivery to the laboratory.) These results also indicate that this natural seminal substrate was produced and stored mainly in the seminal vesicles along with fructose. It should be noted that the pyruvic acid content of the ampullar fluid, in most cases, was several hundred per cent lower than that found in the seminal vesicles (Table 2, Figure 2).

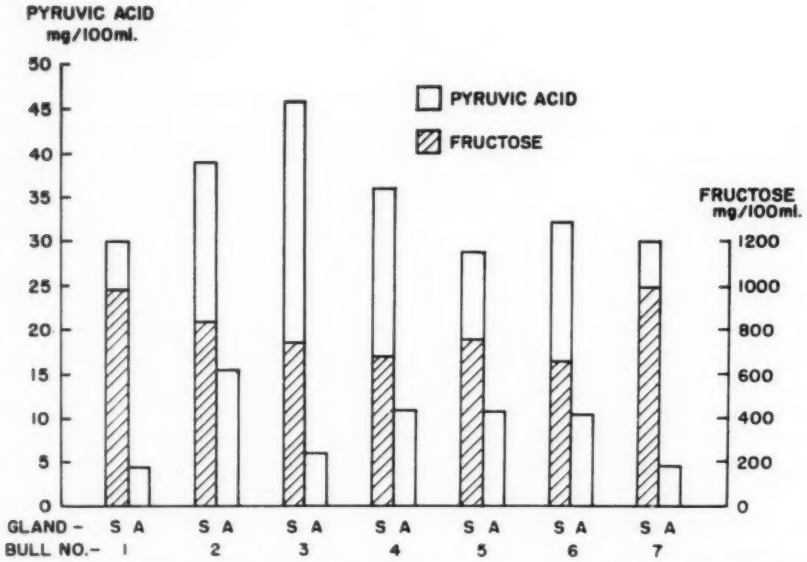


FIG. 2. Pyruvic acid and fructose content of bovine seminal vesicle and ampullar fluids.

7. *Incubation of washed spermatozoa with bovine seminal vesicular fluid and ampullar fluid.* Freshly ejaculated semen was diluted with an equal volume of 0.125 *M* disodium phosphate buffer (pH 7.4). The stoppered tubes were inverted gently three or four times, the samples centrifuged for 20 min. at 1,500 r.p.m., and the supernatant removed. The sperm residue was diluted to the original semen volume with 0.125 *M* disodium phosphate buffer. After resuspension of the cells, the centrifugation was repeated. Finally, the sperm residue was diluted to the original semen volume with phosphate buffer and resuspended by careful stirring.

Two-milliliter aliquots of the resuspended washed spermatozoa mixture were added to samples of seminal vesicular fluid and ampullar fluid, respectively. These were incubated at 37° C. for 60 min., after which the samples were deproteinized and their pyruvic acid content measured quantitatively.

The results indicate that there was indeed a small drop in the pyruvic acid content when seminal vesicular and ampullar fluid were incubated alone (no spermatozoa added), but the rate of utilization was increased many times when washed spermatozoa were added. The concentration of pyruvic acid in the

vesicular fluid fell from 17.9 to 2.7 mg.%, following incubation with washed spermatozoa and, in the case of the ampullar fluid, from 8.5 to 2.5 mg.% (Figure 3).

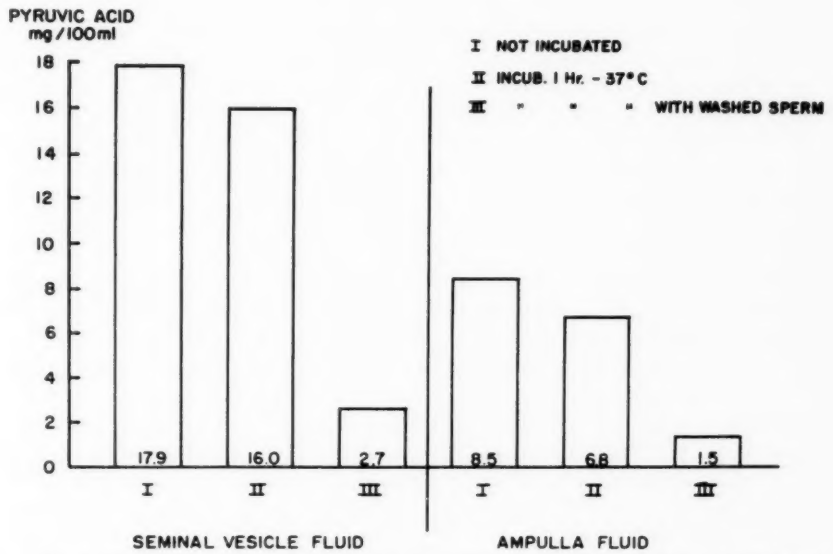


FIG. 3. Effect of washed sperm on the pyruvic acid in fluids from bovine seminal vesicle and ampulla.

Like Stallcup and Hayden (11), our results indicate the presence of lactic dehydrogenase activity in bovine semen, but whereas they report 80% of the activity to be found in the seminal plasma, our results indicate the spermatozoa to be the major source of lactic dehydrogenase activity.

8. *Incubation of bovine seminal vesicular fluid with varying amounts of triphosphopyridine nucleotide (TPN).* It has been well established for nearly 30 yr. that spermatozoa are able to utilize carbohydrate. Fructose disappears and lactic acid is formed. This is believed to be the direct result of glycolysis in which pyruvic acid must play a part, the last reaction in this series of events being the conversion of pyruvic acid to lactic acid, involving the co-enzyme diphosphopyridine nucleotide (DPN).

It was thought that if this was the case, it might be possible to cause an increase in the pyruvic acid content of bovine seminal vesicular fluid if it was incubated with a small amount of DPN. Unfortunately, only TPN was available, but as it can also act as the co-enzyme for this reaction, it was used in this experiment.

Bovine seminal vesicular fluid was obtained as previously described and a 2-ml. aliquot used for each sample. These samples received increasing amounts

of TPN just prior to incubation, after which all the samples, except the control, were incubated for 60 min. at 37° C.

Our solution of TPN was made by taking 2 mg. of TPN, assaying 95%, and dissolving it into 1.07 ml. of phosphate buffer. The three samples received 0.268 mg., 0.53 mg., and 1.07 mg., respectively, of TPN. After incubation, each sample was deproteinized and its pyruvic acid content measured quantitatively.

Our results indicate that there was an increase in the concentration of pyruvic acid on incubation with TPN. If seminal vesicular fluid was incubated alone, there was a decrease in the pyruvic acid content when compared with the unincubated sample. If 1 mg. of TPN was added, the final concentration of pyruvic acid, after incubation, was considerably increased over the unincubated sample.

DISCUSSION

The results presented in this paper can not be reconciled with those of Flerchinger *et al.* (1), who observed that the pyruvic acid content of bovine semen increased on incubation for 1 hr. from approximately 4.5 to 10 mg.%. This increase in pyruvic acid content on incubation is not supported by our data. The method employed by Flerchinger was basically that of Friedmann and Haugen (3) and consisted of making the DNPH derivatives; in this respect it is similar to ours. However, where we have chromatographed our final extract and separated it into many components, Flerchinger measured the total absorption of his final extract with a photoelectric colorimeter. No doubt, Flerchinger was measuring a number of other compounds in addition to pyruvic acid. This conjecture is supported by our results, using paper chromatographic methods by which up to nine separate spots have been obtained from our final extracts, only two of which were derivatives of pyruvic acid.

Our data indicate that pyruvic acid is indeed present in seminal plasma and is stored in the seminal vesicles along with fructose and citric acid at a concentration ranging from 17 to 46 mg.%. Immediately following ejaculation, the concentration of pyruvic acid drops very rapidly to a level so low that it is often barely detectable on a chromatogram. It is considered likely that this endogenous pyruvic acid acts as a source of energy instantly available to the spermatozoa at the moment of ejaculation and is rapidly exhausted, whereas fructose acts as a source of energy over a much longer period of time. The work of Terner (12) would tend to support this hypothesis. He observed that of three C^{14}-labeled substrates studied, pyruvate, acetate, and glucose, washed bovine spermatozoa oxidized pyruvate at the highest rate and glucose at the lowest. We observed only a slight decrease in pyruvic acid content when incubating bovine seminal vesicular fluid alone or, when mixing it with the seminal plasma obtained by centrifugation of a normal ejaculate. In addition, incubation of bovine seminal vesicular fluid with lyophilized whole semen had no effect on the pyruvic acid content. However, when fresh washed spermatozoa were added to seminal vesicular fluid, the pyruvic acid content decreased rapidly from 17.9 to 2.7 mg.% after incubation for 60 min. It appeared that the more

motile the spermatozoa in the sample, the more rapid was the removal of pyruvic acid.

At the present time we do not have any data to indicate what pathways are being utilized by the pyruvic acid during its removal from seminal fluid. If seminal vesicular fluid was incubated with TPN (DPN not being available), an increase in the pyruvic acid content of the fluid was obtained. This may represent a reconversion of some lactate to pyruvic acid. Whether part of the pyruvic acid is utilized by the citric acid cycle awaits elucidation. However, this would be expected and would account for the oxidative phase of sperm metabolism. Flipse and Anderson (2) demonstrated that dialyzed extracts of bovine spermatozoa or seminal plasma were capable of causing a conversion of pyruvate to alanine, and that the reaction could proceed in either direction.

Our data fail to show any correlation between the pyruvic acid and fructose level in the seminal vesicular fluid. As to whether pyruvic acid storage in the seminal vesicles is in any way controlled by the level of androgen output from the testes remains to be investigated. It is of interest to note that we have found an endogenous pyruvic acid content of 9.5 mg.% in human seminal plasma which rapidly disappears on incubation with human spermatozoa.

REFERENCES

(1) Flerchinger, F. H., Erb, R. E., Mikota, L. E., and Ehlers, M. H. Metabolism of Bull Semen. III. Relation of Lactic Acid and Its Accumulation During Incubation with Other Semen Quality Measurements and Nonreturns. J. Dairy Sci., 39: 1006. 1956.

(2) Flipse, R. J., and Anderson, W. R. Metabolism of Bovine Semen. VII. Pyruvate Alanine Conversion. J. Dairy Sci., 42: 637. 1959.

(3) Friedmann, T. E., and Haugen, G. E. The Determination of Keto Acids in the Blood and Urine. J. Biol. Chem., 147: 415. 1943.

(4) Gassner, F. X., and Hopwood, M. L. Seminal Amino Acid and Carbohydrate Pattern of Bulls with Normal and Abnormal Testes Function. Proc. Soc. Exptl. Biol. Med., 81: 37. 1952.

(5) Humphrey, G. F., and Mann, T. Studies of the Metabolism of Semen. Biochem. J., 44: 97. 1949.

(6) Lardy, H. A., and Phillips, P. H. Inhibition of Sperm Glycolysis and Reversibility of the Effect of Metabolic Inhibitors. J. Biol. Chem., 148: 343. 1943.

(7) Mann, T. Studies on the Metabolism of Semen. Fructose as a Normal Constituent of Seminal Plasma. Site of Formation and Function of Fructose in Semen. Biochem. J., 40: 481. 1946.

(8) Melrose, D. R., and Terner, C. The Metabolism of Pyruvate in Bull Spermatozoa. Biochem. J., 53: 296. 1953.

(9) Redenz, E. Über den Spaltungsstoffwechsel der Saugetierspermatozoen in Zusammenhang mit der Beweglichkeit. Biochem. Z., 257: 234. 1933.

(10) Roe, J. H. A Colorimetric Method for the Determination of Fructose in Blood and Urine. J. Biol. Chem., 107: 15. 1954.

(11) Stallcup, O. T., and Hayden, J. S. Lactic Dehydrogenase Activity in Bovine Semen. J. Dairy Sci., 43: 266. 1960.

(12) Terner, C. Effect of Nitrophenols on the Metabolism of Spermatozoa. Federation Proc., 16: 26. 1957.

(13) Towers, G. N., Thompson, J. F., and Steward, F. C. The Detection of Keto Acids of Plants, a Procedure Based on Their Conversion to Amino Acids. J. Am. Chem. Soc., 76: 2392. 1954.

FEMALE AND BREED ASSOCIATED WITH SEASONAL FERTILITY VARIATION IN DAIRY CATTLE [1]

G. H. STOTT

Department of Dairy Science, University of Arizona, Tucson

SUMMARY

Seasonal variation in breeding efficiency was studied, using breeding records of dairy cows inseminated in Arizona with semen collected in three different climatic areas (Palo Alto, California; Columbus, Ohio; and Phoenix, Arizona). Breeding records of cows inseminated outside the state of Arizona at the same time with semen from the same bulls from Palo Alto were used for comparison. Two main seasonal depressions in fertility were found to occur during the year. Pooling data from all breeds (Jerseys, Holsteins, and Guernseys were studied), fertility dropped 3.4 percentage points during the winter and 12.4 percentage points during the hot summer months.

These factors point to the female as the major contributor to the summer depression:

(1) Seasonal depressions in fertility are comparable for all sources of semen used in Arizona.

(2) Cows bred outside of Arizona during the same period and with semen from the same bulls showed a high rate of fertility during the summer months contrasting with the lowered breeding efficiency within the state.

Differences in seasonal fertility were found according to breed, also. The Jerseys show no depression during the summer months, whereas both Guernseys and Holsteins show deep declines in fertility. According to the data, the Guernseys have an equivalent low level during the winter, whereas a significant but somewhat lesser depression occurs for the Holsteins as well.

Numerous studies have shown seasonal differences in breeding efficiency in dairy cattle (2-7, 9, 13, 14, 19, 20). In areas of moderate temperature, light has been implicated as the important factor (4, 6, 13, 14). A lower breeding efficiency occurs in the winter and spring months in these areas corresponding to the shorter daylight periods. In areas where seasonal high temperatures are common, a lower efficiency corresponds with the high ambient temperatures of summer and early fall (2, 3, 7, 9, 20).

Most of the interest and research concerning seasonal effects on breeding efficiency in dairy cattle have been directed toward the male. However, semen quality has not necessarily coincided with observed seasonal fluctuations in fertility as judged by laboratory procedures (1, 5, 6, 9, 10, 15, 18, 21). The possibility that the cow may be important in affecting the seasonal variation has been considered, but there is a lack of published data actually identifying its part.

The objectives of this study were to determine (1) to what extent high environmental temperatures in Arizona depress breeding efficiency, (2) the female's contribution to seasonal variation, and (3) whether there is a breed difference in seasonal fertility.

Received for publication October 6, 1960.

[1] Contribution from Arizona Agricultural Experiment Station, Technical Paper No. 613.

METHODS AND MATERIALS

Through the courtesy of Eads' Breeding Service, Phoenix, Arizona, records for this study were made available. The study covers a 10-yr. period (1948 to 1957 inclusive) of breeding by artificial insemination. The information was confined to cows bred in the Salt River Valley, a uniform climatic area surrounding Phoenix. Most of the animals included in the study were from herds of 50 to 250 in number. However, all the animals in each herd were not necessarily serviced by the breeding organization. Many special matings were made in registered herds. During the last 2 yr., 1956 and 1957, 333 and 340 different herds were represented. The Jersey and Guernsey breeds, being in the minority, had 16 and 22 herds represented, respectively, with additional animals from mixed herds. Four professional inseminators were responsible for the inseminations. There was no conscious attempt for any one inseminator to practice on any one breed.

During the first 8 yr., the semen used was collected and processed at Phoenix. Though no records were maintained on the quality of each collection used for breeding, it was the practice to use only high-quality semen as judged by motility and the number of live spermatozoa. Semen collections were made at least twice weekly on each bull, with no semen used after the third day following collection. During the last 2 yr. the fresh liquid semen was shipped daily from the American Breeders Service at Palo Alto, California, and in addition, frozen semen from Central Ohio Breeders Association, Columbus, Ohio, also was used.

The sources of semen represent three diverse climatic conditions. The average temperature in Phoenix during July is 94° F. (22), with a normally wide daily minimum to maximum change of 30 to 40° F. Most of June, August, and September are comparable. Palo Alto, California, and Columbus, Ohio, as a matter of comparison, have average July temperatures of 67.4 and 74.6° F., respectively (22).

Data for the study were transferred from the breeding records to I.B.M. cards and sorted according to breed, bull, source of semen, date of breeding, and number of services for conception. Five dairy breeds and one beef breed were represented in the insemination records. Only the Guernseys, Jerseys, and Holsteins were significant in number and used in determining breed differences. There were 46,236 records of first services processed. Of these, 20,268 had a second service to settle and 8,995 additional cows had three or more services. A large number of bulls was involved in the breeding records (154 head), resulting in a comparatively small average number of services by month for each bull. It was felt that the small numbers would not justify an attempt to determine bull differences by month. However, as a check, data from some individual bulls which had comparatively large numbers of services over several years were sorted by month and examined. The evidence was that the same seasonal trends existed as when the data from all the bulls were pooled. Consequently, in the present paper all the data have been pooped according to breed or source of semen by month, to insure sufficient numbers. The number of services from

bulls at Ohio is limited, but does show the trend of seasonal variation for breeding efficiency and is, therefore, included.

Conception was determined indirectly from the breeding records on the basis of nonreturn after 5 mo. of the breeding date. The data were tabulated as the per cent of successful first services. The results have been organized on tables, to facilitate showing the comparative differences in breeding efficiency according to season, source of semen, and breed. Statistical appraisal of these differences was made by testing the difference between successive months as follows: When p_1 = the per cent of successful service for the first month with n_1 observations and p_2 = per cent successful service for the second month with n_2 observations: $P = \frac{n_1 p_1 + n_2 p_2}{n_1 + n_2}$ then $s(p_1 - p_2) = \sqrt{(P)\ (1\text{-}P)\ [1/n_1 + 1/n_2]}$ and $t = \frac{P_1 - P_2}{s(p_1 - p_2)}$ can be compared with the "t" value as the 5 and 1% level (11).

When comparatively wide differences occur between months, it is concluded that these differences are significant and are probably due to some seasonal effect. In the included Tables (1 and 2) each per cent successful service is marked with an asterisk when it varies significantly (* $P < 0.05$); with a double asterisk when variation from the preceding month is highly significant (** $P < 0.01$). The same terminology is used in the text in referring to differences in seasonal breeding efficiency.

To determine further the cow effect on seasonal variation in breeding efficiency, 60-90 day nonreturn breeding records of the bulls from Palo Alto were also obtained. These mainly represented inseminations of cattle in the area of northern California and surrounding western states during the same

TABLE 1

Breed by month

Month	Jersey		Guernsey		Holstein	
	No. 1st services	% Non-returns	No. 1st services	% Non-returns	No. 1st services	% Non-returns
January	586	57.5	672	45.5	2973	59.0**
February	505	55.4	548	57.7**	2461	56.7
March	575	59.7	589	58.7	2558	59.3*
April	511	62.0	546	54.8	2278	57.9
May	439	54.9**	604	54.8	2221	61.2*
June	370	57.0	531	54.2	1950	57.4**
July	371	56.6	459	43.6**	1594	47.2**
August	317	57.7	444	46.8	1797	44.4*
September	395	55.7	439	55.4**	2393	46.9
October	357	57.1	540	51.9	2822	56.1**
November	525	56.8	695	57.0*	4697	59.2*
December	628	58.0	734	49.7*	4737	61.9*

* The per cent successful service differs from the previous month by an amount that is significant ($P < 0.05$).

** The per cent successful service differs from the previous month by an amount that is highly significant ($P < 0.01$).

TABLE 2

Source of semen by month

Month	Phoenix		Columbus		Palo Alto		
	Used in Arizona		Used in Arizona		Used in Arizona		Used outside Arizona
	No. 1st services	% Non-returns (5 mo.)	No. 1st services	% Non-returns (5 mo.)	No. 1st services	% Non-returns (5 mo.)	% Non-returns (60-90 days)
January	3,755	56.4*	177	50.8	419	62.3	64.2
February	3,019	56.9	108	48.1	459	57.7	68.6
March	3,237	59.3*	122	60.7	507	57.8	69.7
April	2,894	57.0	117	53.8	465	61.5	70.0
May	2,805	58.4	115	60.9	475	63.8	71.5
June	2,467	55.9*	92	59.8	421	57.2*	71.8
July	2,018	48.7**	83	42.2	383	44.6**	69.3
August	2,051	46.0*	72	41.7	473	50.1*	71.1
September	2,256	49.7*	132	47.7	897	47.7	68.6
October	2,472	55.1**	175	58.9*	1,133	56.3**	69.5
November	4,055	58.4*	213	52.6	1,757	60.8*	66.6
December	4,315	59.3	218	55.2	1,592	62.6	67.4

* The per cent successful service differs from the previous month by an amount that is significant ($P < 0.05$).

** The per cent successful service differs from the previous month by an amount that is highly significant ($P < 0.01$).

period that the semen was being used in Arizona. The data made available were based on the percentage of successful services by month for each bull. The number of services for each bull by month was not given. However, considering the large area serviced by the breeding organization and the popularity of the bulls involved, it is evident that the pooled services for each month would be large in number. Since regular shipments of fresh liquid semen were made to Phoenix, it could be assumed that they were from the same or comparable collections. Seasonal variation in nonreturns from the two sources of data, having used the same semen, should be indicative of the climatic effects on breeding efficiency of cows alone. It is understood, of course, that 60-90 day nonreturn breeding data should show a 10-15% higher rate than 5-mo. nonreturn records (8); however, they should show parallel seasonal variations under comparable conditions. Both have been placed in Table 2 to demonstrate possible differences.

RESULTS AND DISCUSSION

Seasonal variation in breeding efficiency. The per cent of first-service nonreturns by months is shown in Tables 1 and 2. There are two seasonal depressions in breeding efficiency indicated, one occurring during the winter and early spring, the other during the summer and early fall. The depression during the winter and early spring (December to March) varies somewhat in magnitude and time according to breed and source of semen. The Guernseys, being affected more than other breeds during the winter, drop from 57.0% in November to 45.5% (Table 1) in January—an 11.5 percentage point decline. The Holsteins

have a 5.2 percentage point drop from December to February and the Jerseys a 2.6 decline. All the differences indicated, with the exception of the Jerseys, are highly significant. These data are not unlike the findings of Mercier and Salisbury (13, 14), Erb and Waldo (6), and Ehlers and Erb (4), who linked the lowered fertility of winter and early spring with the preceding shorter daylight periods.

Mercier and Salisbury (13, 14) believe that light influences reproductive functions in cattle by its action on the pituitary. Considering the short length of daylight (8.2 hr.) during the winter at the latitudes at which their information was collected (Eastern Canada and New York), these authors suggested that this was the reason that relatively better breeding conditions were found in the winter months in the lower latitudes of the United States where the shortest daylight period is over 10 hr. On a relative basis this might appear to be true in the present data, if only the low summer breeding efficiency is compared with the rest of the year. However, even at the lower latitudes of Southern Arizona a seasonal depression in breeding efficiency comparable to that existing in Eastern Canada, New York, and the State of Washington is demonstrated during the winter months.

The second and most significant seasonal depression in breeding efficiency parallels the high seasonal climatic temperature of Arizona during June, July, August, and September. Where data from all breeds and all sources of semen are pooled there is a decline from May (59.1%) to August (46.7%) of 12.4 percentage points. The Guernsey and Holstein breeds have suppressions of 11.2 and 16.8%, respectively (Table 1). These differences are also highly significant.

Following the longest daylight period of the year, this summer depression contrasts sharply with the results as reported in the northern states and Canada (4, 6, 13, 14). It also contrasts with the 60-90 day nonreturn data for Palo Alto semen used outside of Arizona (Table 2), which show peak breeding efficiency during the summer season. However, it does agree with studies made in other southern climates where lower breeding performance during the summer months has been attributed to high ambient temperatures (2, 3, 5, 7, 20). Whether the semen was produced in Columbus, Palo Alto, or Phoenix (Table 2), the breeding performance during the summer follows the same pattern; thereby indicating that the major seasonal variance in breeding efficiency occurring during the hotter season is contributed by the female.

Breed difference in seasonal variation. Each of the three breeds represented—Holsteins, Guernseys, and Jerseys—shows a different seasonal fertility pattern (Table 1). The Holsteins have peak performances during the months of May (61.2%) and December (61.9%). Each of the two peaks is followed by declining fertility levels in the ensuing months, reaching low points during February (56.7%) and August (44.4%). If light and temperature are the two major factors influencing reproduction, it is not inconceivable that the breeding performance should constantly change from month to month related to the change in length of daylight and temperature. In the Holsteins (Table 1), where a

comparatively large number of first services by month are involved, the difference in successful service from the previous month is statistically significant or highly significant for nearly every month of the year.

The Guernseys also show a month-to-month variation in fertility. However, a deeper depression during the winter months (November 57.0% to January 45.5%), which is below the average of all breeds (55.9%), and comparable decline during July (43.6%) and August (46.8%), would indicate additional variation during these two seasons.

The Jerseys, on the other hand, have but one peak performance. This occurs during March (59.7%) and April (62.0%). Unlike the Guernseys and Holsteins, there are no indications of a depression in breeding efficiency during the summer months. Whether this is related to the apparently greater tolerance of Jerseys to high environmental temperatures (16, 17) is not known, but does indicate a genetic difference in seasonal breeding performance during the hot summer months.

ACKNOWLEDGMENTS

The author is grateful to the following for their willing cooperation and use of breeding records: John and Seth Eads of the Eads Breeding Service, Phoenix, Arizona, and Dr. F. I. Elliott of the American Breeders Service, Madison, Wisconsin.

REFERENCES

(1) Anderson, J. Seasonal Variation in the Reproductive Capacity of the Bull. J. Agr. Sci., 35: 184. 1945.

(2) Branton, C., Griffith, W. S., Hall, J. G., Johnston, J. E., and Patrick, T. E. Breeding Difficulties of Dairy Cattle in Louisiana. Louisiana Agr. Expt. Sta., Ann. Rept. 1954-55.

(3) Branton, C., Griffith, W. S., Norton, H. W., and Hall, J. G. The Influence of Heredity and Environment on the Fertility of Dairy Cattle. J. Dairy Sci., 39: 933. 1956.

(4) Ehlers, M. H., and Erb, R. E. Seasonal Influences on Artificial Breeding with Washington Dairy and Beef Bulls. Proc. West. Div. Am. Dairy Sci. Assoc. 1958.

(5) Erb, R. E., Andrews, F. N., and Hilton, J. H. Seasonal Variation in Semen Quality of the Dairy Bull. J. Dairy Sci., 25: 815. 1942.

(6) Erb, R. E., and Waldo, D. R. Seasonal Changes in Fertility of Dairy Bulls in Northwestern Washington. J. Dairy Sci., 35: 245. 1952.

(7) Erb, R. E., Wilbur, J. W., and Hilton, J. H. Some Factors Affecting Breeding Efficiency in Dairy Cattle. J. Dairy Sci., 23: 549. 1940.

(8) Fosgate, O. T., and Smith, V. R. Prenatal Mortality in the Bovine Between Pregnancy Diagnosis at 34-50 Days Post-Insemination and Parturition. J. Dairy Sci., 37: 1071. 1954.

(9) Fryer, H. C., Marion, G. B., and Farmer, E. L. Nonreturn Rate of Artificially Inseminated Dairy Cows as Affected by Age of Semen, Breed of Bull, and Season. J. Dairy Sci., 41: 987. 1958.

(10) Johnson, J. E., and Branton, C. Effects of Seasonal Climatic Changes on Certain Physiological Reactions, Semen Production, and Fertility in Dairy Bulls. J. Dairy Sci., 36: 934. 1953.

(11) Li, J. C. R. Introduction to Statistical Inference. Published by Jerome C. R. Li, Oregon State University. 1957.

(12) Mercier, E., and Salisbury, G. W. The Effects of Season on the Spermatogenic Activity and Fertility of Dairy Bulls Used in Artificial Insemination. Cornell Vet., 36: 301. 1946.

(13) MERCIER, E., AND SALISBURY, G. W. Seasonal Variations in Hours of Daylight Associated with Fertility Level of Cattle Under Natural Breeding Conditions. J. Dairy Sci., 30: 747. 1947.

(14) MERCIER, E., AND SALISBURY, G. W. Fertility Level in Artificial Breeding Associated with Season, Hours of Daylight, and the Age of Cattle. J. Dairy Sci., 30: 817. 1947.

(15) NAKABAGASHI, N. T., AND SALISBURY, G. W. Factors Influencing the Metabolic Activity of Bovine Spermatozoa. V. Season. J. Dairy Sci., 42: 1806. 1959.

(16) RAGSDALE, A. C., BRADY, S., THOMPSON, H. J., AND WORSTELL, D. M. Environmental Physiology. II. Influence of Temperature, 50° to 105° F., on Milk Production and Feed Consumption in Dairy Cattle. Missouri Agr. Expt. Sta., Research Bull. 425. 1948.

(17) RAGSDALE, A. C., THOMPSON, H. J., WORSTELL, D. M., AND BRADY, S. Environmental Physiology. IX. Milk Production and Feed and Water Consumption Responses of Brahman, Jersey, and Holstein Cows to Changes in Temperature, 50° to 105° F. and 50° to 80° F. Missouri Agr. Expt. Sta., Research Bull. 460. 1950.

(18) SALISBURY, G. W. A Controlled Experiment in Feeding Wheat Germ Oil as a Supplement to the Normal Ration of Bulls Used for Artificial Insemination. J. Dairy Sci., 27: 551. 1944.

(19) SALISBURY, G. W., BRATTON, R. W., AND FOOTE, R. H. The Bull as One Cause of Delayed Returns to Service in Artificial Breeding. J. Dairy Sci., 35: 250. 1952.

(20) SEATH, D. M., AND STAPLES, C. H. Some Factors Influencing the Reproductive Efficiency of Louisiana Herds. J. Dairy Sci., 24: 510. 1941.

(21) SWANSON, E. W., AND HERMAN, H. A. Seasonal Variation in Semen Quality of Some Missouri Dairy Bulls. J. Dairy Sci., 27: 303. 1944.

(22) U. S. Dept. of Commerce Weather Bureau Climatological Data. Annual Summary. Arizona, California: Vol. 63: 13. Ohio: Vol. 64: 13. 1960.

MEASUREMENT OF GENETIC TREND

L. D. VAN VLECK AND C. R. HENDERSON
Department of Animal Husbandry, Cornell University, Ithaca, New York

SUMMARY

A method for estimating trend in genetic merit is described for a population consisting of animals sired by artificial insemination and of animals sired naturally. The contemporary comparison of unselected progeny of an artificially used sire and their naturally sired contemporaries making first records under the same conditions constitutes a unit of observation. This observation consists of an effect of the artificially used sire, an effect due to the random sample of natural service sires with progeny making records in the same year-season, and a random error. This model is fitted by the method of least squares to estimate trend in natural service progeny. Trends in merit of artificially sired daughters and of naturally sired daughters were estimated by this procedure for New York DHIA Holstein herds. From 1951 to 1959 the natural service genetic merit increased by 17 lb. of fat and 399 lb. of milk, whereas the genetic merit of the artificially sired population increased by 25 lb. of fat and 512 lb. of milk. The artificially sired daughters were superior in all seasons for milk and fat yield.

Measurement of the trend in genetic merit separate from environmental trend in dairy cattle populations has interested many groups. Henderson *et al.* (2) presented a procedure for measuring trend in genetic merit apart from environmental trend in the presence of selection. This procedure, however, is extremely complicated computationally and the computing time and costs are prohibitive for large amounts of data. Robertson and Rendel (3) earlier proposed a method for determining the difference between daughters of A.I. (artificial insemination) sires and natural service daughters. The method, commonly described as a contemporary comparison, uses first records only, weighted according to the inverse of the variance of the difference between A.I. and natural service daughters within a herd. If this procedure is modified to make the comparison within a herd-year-season of freshening group, then season and year effects as well as herd effects are removed from the difference. Except for sampling error, this comparison is a measure of the difference in genetic merit between A.I. and non-A.I. daughters. Since this initial report, several investigators have estimated contemporary comparison differences for United States data—Wadell and McGilliard (7) for Michigan herds, Tucker and Farthing (4) and Tucker *et al.* (5) for North Carolina data, and Hahn *et al.* (1) for Georgia herds. VanVleck and Henderson (6), working with New York Holstein records, reported differences between A.I. and non-A.I. daughters over a 9-yr. period.

Perhaps of more interest to the animal breeder is the trend in genetic merit in the entire population, a population which consists of both A.I. and non-A.I. daughters. The purpose of this paper is to present a procedure which measures the change in merit of non-A.I. daughters, which then allows measurement of the change in merit of the A.I. population.

Received for publication April 1, 1961.

EXPERIMENTAL PROCEDURE

First, consider the contemporary comparison of Robertson and Rendel (3) within a given herd-year-season for daughters of a particular sire. Assume that the records are adjusted to a mature equivalent basis, that there was no selection on these animals prior to the initiation of the first record, and that the records of cows culled before completion of a record are extended to a 305-day equivalent. Under these assumptions, the underlying model for an age-corrected 305-day record is

$$Y_{ijkp} = \mu + h_i + s_j + m_k + e_{ijkp} \qquad (1)$$

Where Y_{ijkp} is the record made in the i^{th} herd in the k^{th} year-season of freshening by the p^{th} first-lactation cow sired by the j^{th} sire, μ is a constant population parameter, s_j is an effect due to the j^{th} sire, h_i is an effect due to the i^{th} herd, m_k is an effect due to the k^{th} year-season, and e_{ijkp} is a random error common to the $ijkp^{th}$ record. The s_j are considered fixed effects and the h_i and m_k either fixed or random effects. It can be verified that interaction between herd and year-season effects would be permissible, since comparisons will be made within herd-year-season groups but not interactions involving sire effects. The e_{ijkp} are independently and identically distributed with mean zero and variance σ^2.

The contemporary comparison between A.I. and non-A.I. daughter averages within a herd-year-season for a particular A.I. sire is

$$d_{ijk} = y_{1ijk.} - y_{2ijk.};$$

where $y_{1ijk.}$ is the mean of n_{1ijk} daughters of the j^{th} A.I. sire in the i^{th} herd in the k^{th} year-season and $y_{2ijk.}$ is the mean of n_{2ijk} non-A.I. daughters in the same herd-year-season. The variance of this difference is

$$V(d_{ijk}) = \frac{n_{1ijk} + n_{2ijk}}{n_{1ijk}n_{2ijk}}\sigma^2.$$

The weighted contemporary comparison for a sire in a particular year-season is $d_{.jk} = \dfrac{\sum_i w_{ijk}d_{ijk}}{\sum_i w_{ijk}}$; where w_{ijk} is the inverse of the variance of d_{ijk}. In terms of the model (1) the expected value of the difference is

$$E(d_{.jk}) = s_j - s_k^* \text{ with variance } V(d_{.jk}) = \frac{\sigma^2}{\sum_i w_{ijk}} = \frac{\sigma^2}{w_{.jk}}.$$

It is noted that this weighted comparison expresses the difference between the effect of the j^{th} A.I. sire, s_j, and an average sire effect of non-A.I. daughters in the k^{th} year-season, s_k^*. The s_k^* is a measure of the average genetic merit of natural service sires having daughters in that year-season of freshening. The s_j is a measure of the genetic merit of the j^{th} A.I. sire.

Now take the $d_{.jk}$ as units of observations. Under the assumptions that the sires of non-A.I. daughters contemporary to daughters of an A.I. sire are a random sample of non-A.I. sires whose daughters have records in that particular year-season, and that A.I. sires and non-A.I. sires are mated to a random sample of cows, the least-squares equations for estimating the s_j and s_k^* are:

$$\sum_j w_{.jk}s_j - \sum_j w_{.jk}s_k^* = \sum_j w_{.jk}d_{.jk} \ (j = 1, \ldots, J) \text{ and}$$

$$-\sum_k w_{.jk}s_j + \sum_k w_{.jk}s_k^* = \sum_k w_{.jk}d_{.jk} \ (k = 1, \ldots, K).$$

In matrix notation the equations become

$$\begin{bmatrix} D & C \\ C' & A \end{bmatrix} \begin{bmatrix} B_1 \\ B_2 \end{bmatrix} = \begin{bmatrix} Y_1 \\ Y_2 \end{bmatrix} \qquad \text{where}$$

D is a known J^2 diagonal matrix, A is a known K^2 diagonal matrix, C is a known $J \times K$ matrix, B_1 is a $J \times 1$ vector of unknowns (A.I. sire effects), B_2 is a $K \times 1$ vector of unknowns (year-season effects of non-A.I. sires), Y_1 is a known $J \times 1$ vector, and Y_2 is a known $K \times 1$ vector. A restriction is required in order to solve these equations. A convenient restriction is $s_K^* = 0$. A set of $J + K - 1$ equations in $J + K - 1$ unknowns remains. Usually, J will be large and K relatively small.

Following the usual procedure for absorbing the largest group of equations, the estimates for the genetic merit of non-A.I. sires in the $K - 1$ year-seasons may be obtained as follows:

$$B_2 = S^{-1}Z \qquad \text{where}$$
$$S = A - C'D^{-1}C \text{ and}$$
$$Z = Y_2 - C'D^{-1}Y_1 .$$

The solutions B_2 provide an estimate of genetic trend in the non-A.I. population for a period of K year-seasons relative to Year-Season K, since the estimate for Year-Season K was set equal to zero. The sampling variance-covariance matrix of B_2 is $\sigma^2 S^{-1}$.

The absorbed A.I. sire effects can be estimated as $B_1 = D^{-1}(Y_1 - CB_2)$. It should be noted that the estimate of an A.I. sire effect is corrected for the average non-A.I. sire effects for the year-seasons in which contemporary comparisons were made for his daughters, thus correcting for any genetic trend in the non-A.I. population.

The contemporary comparison procedure of Robertson and Rendel (3), which compares all A.I. and non-A.I. first-lactation daughters in the same herd-year-season, estimates the superiority of A.I. progeny over their non-A.I. contemporaries. An estimate of the trend in genetic merit of the A.I. population can be constructed by adding the seasonal estimates of contemporary comparison superiority to the estimates of trend in the non-A.I. population. The estimates of A.I. superiority and non-A.I. trend are not independent, since the same non-A.I. daughters appear in both sets of estimates, as do the A.I. daughters.

An example may clarify the procedure. First, computation of a comparison for the daughters of the first A.I. sire with their contemporaries beginning records in Herd One and Year-Season One is as follows: Suppose that the mean of two daughters of the sire is 15 and that the mean of three contemporary non-A.I. daughters is ten. Then $d_{111} = 15 - 10 = 5$ and $w_{111} = \dfrac{2 \times 3}{2 + 3} = \dfrac{6}{5}$. Next,

suppose $d_{211} = 10$ and $w_{211} = \frac{1 \times 3}{1+3} = \frac{3}{4}$; $d_{311} = 5$ and $w_{311} = \frac{1 \times 2}{1+2} = \frac{2}{3}$, and the sire has no other comparisons in Season One. This gives $d_{.11} = \left(\frac{6}{5} \times 5 + \frac{3}{4} \times 10 + \frac{2}{3} \times 5\right) \Big/ \left(\frac{6}{5} + \frac{3}{4} + \frac{2}{3}\right) = 6.43$ and $w_{.11} = 2.62$.

Continuing the example, suppose that there are three sires and four year-seasons with subclass totals, $d_{.jk}$'s, and subclass weights, $w_{.jk}$'s, as given in Table 1.

TABLE 1

Subclass totals and weights for a small example.[a]

Sire	Year-season 1	2	3	4	$\sum_k w_{.jk} d_{.jk}$
1	6.43(2.62)	7.00(3.00)	7.50(2.50)	0	56.60
2	0	5.25(1.75)	6.66(1.33)	8.00(3.33)	44.69
3	4.00(1.00)	6.00(2.00)	0	5.00(1.33)	22.65
$\sum_j w_{.jk} d_{.jk}$	20.85	42.19	27.61	33.29	

[a] The first entry of each cell is the sire by year-season subclass total, $d_{.jk}$, and the following entry in parentheses is the subclass weight, $w_{.jk}$).

The least-squares equations for this example are:

$$\left\{\begin{array}{rrr|rrrr} 8.12 & 0 & 0 & -2.62 & -3.00 & -2.50 & 0 \\ 0 & 6.41 & 0 & 0 & -1.75 & -1.33 & -3.33 \\ 0 & 0 & 4.33 & -1.00 & -2.00 & 0 & -1.33 \\ \hline -2.62 & 0 & -1.00 & 3.62 & 0 & 0 & 0 \\ -3.00 & -1.75 & -2.00 & 0 & 6.75 & 0 & 0 \\ -2.50 & -1.33 & 0 & 0 & 0 & 3.83 & 0 \\ 0 & -3.33 & -1.33 & 0 & 0 & 0 & 4.66 \end{array}\right\} \left\{\begin{array}{c} S_1 \\ S_2 \\ S_3 \\ \hline S_1^* \\ S_2^* \\ S_3^* \\ S_4^* \end{array}\right\} = \left\{\begin{array}{r} 56.60 \\ 44.69 \\ 22.65 \\ \hline 20.85 \\ 42.19 \\ 27.61 \\ 33.29 \end{array}\right\}$$

The D matrix is the upper left 3×3 matrix, the A matrix is the lower right 4×4 matrix, and the C matrix is the upper right 3×4 matrix. The B_1 vector of unknowns corresponds to the S's and the B_2 vector of unknowns to the S^*'s. Y_1 and Y_2 make up the vectors of sire and year-season totals. Note that one restriction must be imposed in order to solve the equations, since the last four rows add up to the first three.

The solutions to the equations for S_1^*, S_2^*, and S_3^* are estimates of the genetic merit of non-A.I. sires in Year-Seasons One, Two, and Three relative to Year-Season Four. The estimated increase from Year-Season One to Year-Season Four is given by $\hat{S}_4^* - \hat{S}_1^* = 0 - \hat{S}_1^*$. The solutions for S_1, S_2, and S_3 are estimates of the genetic merit of A.I. sires corrected for the merit of their non-A.I. contemporaries. For example, the estimate of S_1 is $\hat{S}_1 = \frac{1}{8.12}(56.60 + 2.62\,\hat{S}_1^* + 3.00\,\hat{S}_2^* + 2.50\,\hat{S}_3^*)$ and of S_2 is $\hat{S}_2 = \frac{1}{6.41}(44.69 + 1.75\,\hat{S}_2^* + 1.33\,\hat{S}_3^*)$, remembering that $\hat{S}_4^* = 0$.

Application of the method to a New York Holstein population. Contemporary comparisons of daughters of 236 Holstein A.I. sires by year-season of first

freshening were obtained from mature equivalent 305-day corrected milk and fat records available at the New York Dairy Records Processing Laboratory. The year-seasons of freshening as used by the Laboratory are given in Table 2.

TABLE 2
Year-seasons of calving used in the New York Dairy Records Processing Laboratory

Year-season	Inclusive months	Year-season	Inclusive months
50-2	September 1950—February 1951	56-1	March 1956 —August 1956
51-1	March 1951 —August 1951	56-2	September 1956—February 1957
51-2	September 1951—February 1952	57-1	None
52-1	March 1952 —August 1952	57-2	March 1957 —July 1957
52-2	September 1952—February 1953	57-3	August 1957 —November 1957
53-1	March 1953 —August 1953	58-1	December 1957 —March 1958
53-2	September 1953—February 1954	58-2	April 1958 —July 1958
54-1	March 1954 —August 1954	58-3	August 1958 —November 1958
54-2	September 1954—February 1955	59-1	December 1958 —March 1959
55-1	March 1955 —August 1955	59-2	April 1959 —July 1959
55-2	September 1955—February 1956	59-3	August 1959 —November 1959

The records were the same as those investigated by VanVleck and Henderson (6). Since the number of comparisons was small in Year-Seasons 50-2, 51-1, and 51-2, the contemporary comparisons in those year-seasons were pooled for each A.I. sire group. Counting this as one year-season, there was a total of 19 year-seasons in the 9-yr. period. Most sires did not have contemporary comparisons in every year-season. Some appeared in only one year-season.

The restriction $S_{19}^* = 0$ was imposed and the equations solved by absorbing the A.I. sire equations as described. Estimates of the non-A.I. genetic trend appear in Table 3. To follow the values more easily, the estimates for 1951

TABLE 3
Estimated genetic trend in the naturally sired Holstein population and in the artificially sired Holstein population of New York

Year-season	Fat production *(lb.)*			Milk production *(lb.)*		
	non-A.I. trend	A.I. superiority	A.I. trend	non-A.I. trend	A.I. superiority	A.I. trend
1951	0.00	9.92	9.92	0.0	262.3	262.3
52-1	1.25	9.10	10.35	226.5	72.9	299.4
52-2	0.47	5.58	6.05	85.0	56.0	141.0
53-1	8.59	0.62	9.21	231.9	2.7	234.6
53-2	6.67	4.03	10.70	215.1	14.1	229.2
54-1	6.16	9.89	16.05	140.7	255.3	396.0
54-2	8.24	10.38	18.62	140.3	294.3	434.6
55-1	5.13	16.51	21.64	32.9	461.2	494.1
55-2	14.91	9.24	24.15	286.4	278.6	565.0
56-1	8.34	9.61	17.95	213.6	132.5	346.1
56-2	9.49	12.31	21.80	264.2	183.3	447.5
57-2	15.49	8.44	23.93	302.8	227.9	530.7
57-3	12.52	8.43	20.95	193.8	186.9	380.7
58-1	15.36	8.57	23.93	355.3	87.6	442.9
58-2	16.26	5.07	21.33	456.2	17.5	473.7
58-3	16.08	11.08	27.16	349.6	212.2	561.8
59-1	10.71	14.07	25.42	272.3	235.8	508.1
59-2	5.82	19.84	25.66	117.2	441.2	558.4
59-3	17.15	18.19	35.34	399.2	375.4	774.6

were changed to zero and the other values changed accordingly by addition of 17.15 for fat and 399.2 for milk. The contemporary comparison estimates of the superiority of A.I. daughters over non-A.I. daughters, as compared earlier by VanVleck and Henderson (6), are also given by year-season in Table 3. The estimated trend in A.I. merit obtained by adding the estimates of non-A.I. trend and A.I. superiority is also shown in Table 3.

There has been a small but relatively steady increase in genetic merit for milk and fat production in both the A.I. and non-A.I. populations over the 9-yr. period. The increase has been nearly 48% greater for the A.I. progeny than for the non-A.I. progeny for fat production and about 28% for milk production. The fluctuations from one year-season to another are greater for milk production than for fat production, although the trends for milk and fat are generally the same. The rate of increase is slightly greater for fat than for milk in both populations and the spread between populations increases faster for fat production. This situation was probably caused by the greater emphasis on fat production than on milk production in selection of sires.

REFERENCES

(1) Hahn, E. W., Carmon, J. L., and Miller, J. W. An Intra-Herd Contemporary Comparison of the Production of Artificially and Naturally Sired Dairy Cows in Georgia. J. Dairy Sci., 41: 1061. 1958.

(2) Henderson, C. R., Kempthorne, O., Searle, S. R., and vonKrosigk, C. M. The Estimation of Environmental and Genetic Trends from Records Subject to Culling. Biometrics, 15: 192. 1959.

(3) Robertson, A., and Rendel, J. M. The Performance of Heifers Got by Artificial Insemination. J. Agr. Sci., 44: 184. 1954.

(4) Tucker, W. L., and Farthing, B. R. Genetic Improvement of Dairy Cattle Attributable to Sires Used in Artificial Insemination in North Carolina. J. Dairy Sci., 41: 740. 1958.

(5) Tucker, W. L., Legates, J. E., and Farthing, W. L. Genetic Improvement in Production Attributable to Sires Used in Artificial Insemination in North Carolina. J. Dairy Sci., 43: 982. 1960.

(6) VanVleck, L. D., and Henderson, C. R. Improvement in Production of New York Holsteins Due to Artificial Insemination. J. Dairy Sci., 44: 1328. 1961.

(7) Wadell, L. H., and McGilliard, L. D. Influence of Artificial Breeding on Production in Michigan Dairy Herds. J. Dairy Sci., 42: 1079. 1959.

MILK REPLACERS FOR DAIRY CALVES. I. A COMPARISON OF AN ALL MILK BY-PRODUCT REPLACER AND LIMITED WHOLE MILK WITH AND WITHOUT CHLORTETRACYCLINE [1]

F. R. MURDOCK AND A. S. HODGSON
Western Washington Agricultural Experiment Station, Puyallup
AND
T. H. BLOSSER
Department of Dairy Science, Washington Agricultural Experiment Station, Pullman

SUMMARY

Forty Holstein calves, 20 males and 20 females, were used in a 2 × 2 × 2 fractorial design to compare growth response and the incidence and severity of scours of calves fed limited whole milk or milk replacer and each fed with and without chlortetracycline. The milk replacer was formulated entirely from milk by-products, except for fat-soluble vitamin supplementation.

Whole milk intake ranged from 8 to 12 lb. and milk replacer from 0.8 to 1.5 lb/calf/day to weaning age of 7 wk. Chlortetracycline intake varied with feed intake and ranged from 50 to 275 mg/calf/day.

Although normal growth was sustained by both milk and replacer rations, weight gains were significantly greater at 90 days of age for calves fed whole milk. No differences in body weight gain were evident at weaning age. Increases in height at withers or heart girth were not affected by type of milk fed.

Chlortetracycline resulted in significant increases in weight gains at weaning age. However, by 90 days no differences in weight response to antibiotic feeding were evident. The incidence and severity of scours were increased by replacer feeding, and chlortetracycline did not decrease them.

Many dairymen find that feeding whole milk to dairy calves is not economical, because of the high value of fluid milk for human consumption. Consequently, these dairymen are raising herd replacements on some form of milk replacer.

Good, healthy calves can be grown without large amounts of whole milk (1). Williams and Knodt (8) developed a milk replacement formula which produced calves equal in growth and general appearance to milk-fed calves. This formula contained 50% of dried skimmilk, 10% dried whey, and the remainder was of nonmilk sources. Wallace *et al.* (7) found that calves raised on replacement mixtures containing appreciable amounts of substitutes were rougher in appearance and grew more slowly during the first month than whole milk–fed calves. Reid *et al.* (6) and Lassiter (2) have reviewed the effects of antibiotics on growth and well-being of dairy calves. In general, supplemental antibiotics seem to have stimulated growth and reduced the incidence of scours. The growth-promoting value of antibiotics appeared to be greater during early life than during late calfhood.

Received for publication April 24, 1961.

[1] Scientific Paper No. 2107, Washington Agricultural Experiment Station, Pullman. Project 1178.

Since some dairymen in surplus milk-producing areas have felt that using replacers containing nonmilk products would contribute to dried milk surpluses, an all-milk formula (except for vitamin supplements) was developed. This paper reports experimental work comparing performance of calves fed on an all-milk product replacer with calves fed limited whole milk. Each group was fed with and without supplemental antibiotics.

EXPERIMENTAL PROCEDURE

Forty Holstein calves, 20 male and 20 female, were used in this study. Calves were randomly assigned, over a 16-mo. period, by sex, to whole milk or milk replacer and to chlortetracycline or no chlortetracycline at three days of age. Thus, 20 calves, equally divided as to sex, received whole milk and 20 received milk replacer. Likewise, 20 calves, equally divided as to sex and type of milk fed, received no antibiotic, and 20 received antibiotic. Chlortetracycline was added to both the dry milk replacer and to the calf starter for the antibiotic-fed groups at a level of 50 mg/lb. For calves receiving whole milk with antibiotic, chlortetracycline was added at a level of 6.25 mg/lb of milk immediately before feeding. Antibiotic consumption varied with age and feed intake and ranged from 50 to 275 mg. per calf per day. All calves were raised in individual pens, either in a conventional closed calf barn (eight calves) or in an open-shed unit (32 calves). These differences in housing management were uniformly applied over sex, type of milk, and antibiotic treatments.

The milk replacer used in this study consisted entirely of dried milk products, except for small amounts of vitamin A and D supplements. The formula was: dried skimmilk, 45.0%; dried whey, 30.0%; dried sweet cream buttermilk, 24.68%; irradiated yeast (9,000 USP units/g), 0.10% and stabilized vitamin A (20,000 USP units/g), 0.22%. (For future reference this formula will be referred to as WSU No. 2.) The milk replacer was reconstituted with water (see Table 1) and fed at a temperature of about 100° F. This liquid feeding schedule for the milk replacer and whole milk groups is shown in Table 1. All calves were weaned at 7 wk. of age.

Fair-quality, chopped alfalfa hay was fed ad libitum to both groups throughout the experiment. A pelleted calf starter of the following formula (in pounds) was fed: ground corn, 20.0; ground barley, 8.0; ground oats, 20.0; linseed oil

TABLE 1
Milk and replacer feeding schedule

Days of age	Frequency of feeding	Replacer group			Whole milk group
		Milk	Replacer	Water	Milk
		(lb.)			
0–3	With dam	Ad lib.	0.0	0.0	Ad lib.
4–5	Twice daily	4.0	0.0	0.0	4.0
6–10	Twice daily	2.0	0.4	3.0	5.0
11–28	Twice daily	0.0	0.7	5.5	5.5
29–42	Twice daily	0.0	0.75	6.0	6.0
43–49	Once daily	0.0	0.75	6.0	6.0

meal, 25.0; wheat mixed feed, 10.0; dried skimmilk, 10.0; dehydrated alfalfa, 5.0; steamed bone meal, 1.0; iodized salt, 1.0; irradiated yeast (9,000 USP units/g), 0.05. Calves were limited to a maximum of 4 lb. daily. This level of starter intake was not reached, however, until about the time the calves were weaned. All calves were kept on experiment until 90 days of age.

Representative samples of milk replacer, calf starter, and hay were collected weekly. These weekly samples were combined and subsampled for proximate analyses.

Calves were weighed and measured for height at withers and heart girth at three days of age, and at regular weekly intervals. The daily intake of milk or replacer was recorded. Hay and calf starter consumption were tabulated by weekly periods. Any refusal of milk or milk replacer was recorded and the amount refused was taken into account in determining total feed intake. Fresh, clean water was made available to the calves at all times.

Growth and feed consumption data were summarized from zero to 49 days (weaning age) and from three to 90 days.

RESULTS AND DISCUSSION

The gross chemical analyses of the feeds are shown in Table 2.

TABLE 2
Gross chemical composition of feeds (as-fed basis)

Feed	Moisture	Crude protein	Crude fiber	Ether extract	Ash	N.F.E.
Whole milk [a]	87.2	3.5	0.0	3.7	0.7	4.9
Milk replacer	6.0	28.5	0.0	0.5	7.5	57.5
Calf starter	9.5	19.0	6.7	3.7	6.0	55.1
Alfalfa hay	13.0	10.8	35.2	1.3	6.8	32.9

[a] From Morrison's Feeds and Feeding, 22nd edition.

Feed and TDN consumption. The results of an analysis of variance indicated significant differences in starter consumption between sexes ($P < 0.05$) at 49 as well as 90 days of age. Also, highly significant differences in starter consumption ($P < 0.01$) were found between types of milk fed at both ages. Replacer-fed calves consumed the larger amount. This might be anticipated, inasmuch as whole milk–fed calves consumed about 8% more milk solids and because whole milk has a higher energy value. No differences in starter consumption were associated with antibiotic feeding.

The average feed consumption and estimated TDN intakes by treatment groups are shown in Table 3 for both sexes from three to 49 days (weaning age) and three to 90 days of age.

Hay consumption was not significantly ($P > 0.05$) affected by any of the treatments.

Differences in estimated TDN consumption ($P < 0.01$) were evident between types of milk fed at 49 days of age, but no differences were found at 90 days. The higher TDN consumption by milk-fed calves is attributable entirely to

TABLE 3

Average feed and estimated TDN consumption of calves at 49 and 90 days of age

	Male				Female				Error mean square
	Chlortetracycline		No Chlortetracycline		Chlortetracycline		No Chlortetracycline		
	Milk	Replacer	Milk	Replacer	Milk	Replacer	Milk	Replacer	
Whole milk *(lb.)*	468.7	35.9	474.2	35.4	475.5	35.5	466.4	37.7	
Milk replacer *(lb.)*	.....	54.7	.....	55.2	.....	54.6	.....	53.4	
Starter *(lb.)*									
3 to 49 days	50.8	68.6	42.7	50.7	35.2	49.7	31.6	50.1	184.97
3 to 90 days	210.6	229.0	198.4	205.2	177.3	205.2	184.2	208.8	383.10
Hay *(lb.)*									
3 to 49 days	19.9	25.5	20.9	22.1	23.3	28.9	22.9	14.4	118.97
3 to 90 days	143.3	140.9	142.7	130.5	126.9	144.5	124.4	107.0	986.52
Estimated TDN *(lb.)*									
3 to 49 days	123	113	119	99	115	101	110	93	107
3 to 90 days	301	287	292	265	269	271	272	225	524

differences in energy between the whole milk and milk replacer, since the latter group consumed more starter. The replacer-fed calves continued to consume more starter after weaning, to the extent that no differences in estimated TDN were evident at 90 days of age. Estimated TDN consumption by male calves was higher than that of females ($P < 0.05$), both at 49 and 90 days of age. This is logical because of the greater live weight of male calves.

Growth performance. Average increases in body weight, height at withers, and heart girth with their respective error mean squares are given in Table 4.

TABLE 4

Average gains in weight, height at withers, and heart girth by calves at 49 and 90 days of age

	Male				Female				Error mean square
	Chlortetracycline		No Chlortetracycline		Chlortetracycline		No Chlortetracycline		
	Milk	Replacer	Milk	Replacer	Milk	Replacer	Milk	Replacer	
No. of calves	5	5	5	5	5	5	5	5	
Av. weight gains *(lb.)*									
3 to 49 days	79.9	76.6	67.5	63.9	65.7	65.1	63.0	56.1	71.61
3 to 90 days	170.4	148.0	152.1	140.1	134.9	130.9	136.9	127.6	177.23
Av. withers height gain *(in.)*									
3 to 49 days	3.4	2.6	3.2	3.3	3.3	3.3	3.3	3.0	0.41
3 to 90 days	6.8	5.6	6.1	5.9	5.9	5.9	6.3	5.8	0.67
Av. heart girth gain *(in.)*									
3 to 49 days	6.4	5.9	5.3	5.3	5.8	5.5	5.3	5.2	0.80
3 to 90 days	11.1	10.9	10.5	9.9	10.0	10.0	10.0	9.6	0.69

Differences in body weight gains between sex groups at 49 and 90 days of age were significant ($P < 0.01$), with males making the more rapid gains. Antibiotic-fed calves gained significantly more ($P < 0.01$) from three to 49 days of age, but at 90 days of age differences were not significant ($P > 0.05$). This is in agreement with results previously reported from this station (5). Although no significant differences ($P > 0.05$) in weight gains were evident between whole milk and milk replacer–fed calves at weaning age (49 days), highly significant ($P < 0.01$) differences were found at 90 days. The whole milk–fed calves made the more rapid gains during this period.

The apparent discrepancies between estimated TDN intake and rate of weight gain by periods are difficult to explain. There may have been some carry-over effect of the whole milk ration that affected rate of gain after weaning. Another possible explanation is that the TDN values reported by Morrison (4) for milk and other feeds used in this study are not applicable in evaluating rations for young dairy calves.

No significant differences ($P > 0.05$) in height at withers were attributable to sex, antibiotic, or feed treatments.

Male calves gained significantly more in heart girth ($P < 0.01$) at 90 days of age than did females. Also, chlortetracycline-fed calves gained significantly more in heart girth ($P < 0.01$) by weaning age than did those not receiving this antibiotic.

Although some differences in rates of growth were observed due to treatments as indicated above, all calves but one grew normally, in that their rates of growth equalled or exceeded standards as established by Matthews and Fohrman (3). One heifer calf receiving replacer without antibiotic did not quite meet this growth standard.

Observations of incidence and severity of scouring are summarized in Table 5. Twenty-two calves, 11 males and 11 females, scoured on one or more days during the 90 days on experiment. Scouring seemed to occur more frequently and with more severity, as indicated by days of scouring and cases requiring treatment, in calves fed milk replacer than in those fed whole milk.

TABLE 5

Summary of incidence of severity of scouring in calves fed whole milk and replacer with and without antibiotics during 90-day period

	Male				Female			
	Chlortetracycline		No Chlortetracycline		Chlortetracycline		No Chlortetracycline	
	Milk	Replacer	Milk	Replacer	Milk	Replacer	Milk	Replacer
No. of calves	5	5	5	5	5	5	5	5
Cases of scouring	1	7	5	5	2	9	3	6
Days of scouring	1	13	14	12	3	21	5	13
Cases requiring treatment	0	1	0	0	1	3	0	2
Days under treatment	0	1	0	0	1	6	0	3

There appeared to be little if any difference in the incidence or severity of scouring between calves given antibiotic and controls. However, the antibiotic seemed to be more effective in controlling scours in whole milk–fed calves than in those fed replacer.

The scouring data were not considered of sufficient volume or of normal distribution to warrant statistical analysis.

No calves died during the experiment.

ACKNOWLEDGMENTS

The authors thank the Washington State Dairy Products Commission, Seattle, Washington, for their financial assistance; Consolidated Dairy Products Co., Seattle, Washington, for providing the dried milk products; Lederle Laboratories Division of American Cyanamid Company, New York, New York, for the Chlortetracycline, and Nopco Chemical Company, Portland, Oregon, for the stabilized vitamin A used in this study. Thanks are also extended to the Western State Hospital, Ft. Steilacoom, Washington, for providing some of the heifer calves used in this study.

REFERENCES

(1) Converse, H. T. Experiments in Rearing Calves Without Whole Milk and with Limited Amounts of Skimmilk. USDA, Circ. 822. 1949.

(2) Lassiter, C. A. Antibiotics as Growth Stimulants for Dairy Cattle: A Review. J. Dairy Sci., 38: 1102. 1955.

(3) Matthews, C. A., and Fohrman, M. H. Beltsville Growth Standards for Holstein Cattle. USDA, Tech. Bull. 1099. 1954.

(4) Morrison, F. G. Feeds and Feeding. 22nd ed. The Morrison Publishing Company, Ithaca, N. Y. 1956.

(5) Murdock, F. R., Hodgson, A. S., and Blosser, T. H. The Effect of Antibiotics on the Growth and Well-Being of Dairy Calves. Proc. 32nd Ann. Meet. American Dairy Science Association, Western Division. 1951.

(6) Reid, J. T., Warner, R. G., and Loosli, J. K. Feed Supplements: Antibiotics in the Nutrition of Ruminants. J. Agr. and Food Chem., 12: 186. 1954.

(7) Wallace, H. D., Loosli, J. K., and Turk, K. L. Substitutes for Fluid Milk in Feeding Dairy Calves. J. Dairy Sci., 34: 256. 1951.

(8) Williams, J. B., and Knodt, C. B. The Value of Milk Replacements in the Rations of Dairy Calves. J. Dairy Sci., 32: 986. 1949.

CHANGES IN BODY WEIGHT DURING THE FIRST PREGNANCY FOR HOLSTEIN HEIFERS CALVING AT DIFFERENT SEASONS[1]

A. B. SCHULTZE AND H. P. DAVIS
University of Nebraska, Lincoln

SUMMARY

Body weight gains during pregnancy were studied for 100 Holstein heifers calving at different seasons of the year. The year was divided into four seasons as follows: January, February, and March; April, May, and June; July August, and September; and October, November, and December. Body weight gain during gestation was least for that group of heifers calving during the period July, August, and September. Weight gain for the heifers calving during the other three seasons was significantly higher. The weight gain during the last 2 mo. of pregnancy appeared to be positively correlated with subsequent milk yield, regardless of season of calving.

Several reports have been made on body weight changes during pregnancy in dairy cows (4, 5). These reports have been on cows calving throughout the year, regardless of season. Since high summer temperatures suppress physiological function (1) (growth rate, milk production, etc.), gain in weight during pregnancy also may be affected. Season of calving has a marked effect on total milk yield during the following lactation (3). Milk secretion and growth are physiologically related, since the growth hormone is important for both. The season of calving may affect subsequent milk yield by its influence on growth hormone production.

METHODS

Body weights of Holstein heifers were taken monthly before breeding and throughout the first pregnancy. Towards the end of pregnancy and for a variable number of days before calving, body weights were taken daily. Following calving, body weights were again taken daily for a period of ten days, then at monthly intervals thereafter. Weight at breeding was calculated from daily gains made between the monthly weights taken before and after breeding. None of the heifers received pasture in any amount. Monthly weights from breeding to parturition for 25 heifers for each of four seasons are included in this study. The four seasons are divided by months as follows: (a) January, February, March; (b) April, May, June; (c) July, August, September; (d) October, November, December.

To determine whether body weight changes during pregnancy with season of calving was affected by body weight of the heifer at breeding time, the ten heaviest and ten lightest heifers at breeding time for each season were grouped and the variance of groups $\times$ season of calving $\times$ monthly weights was determined.

RESULTS

Body weight changes during pregnancy and for several months following calving are presented in Figure 1. From inspection of this figure and by refer-

Received for publication January 19, 1961.

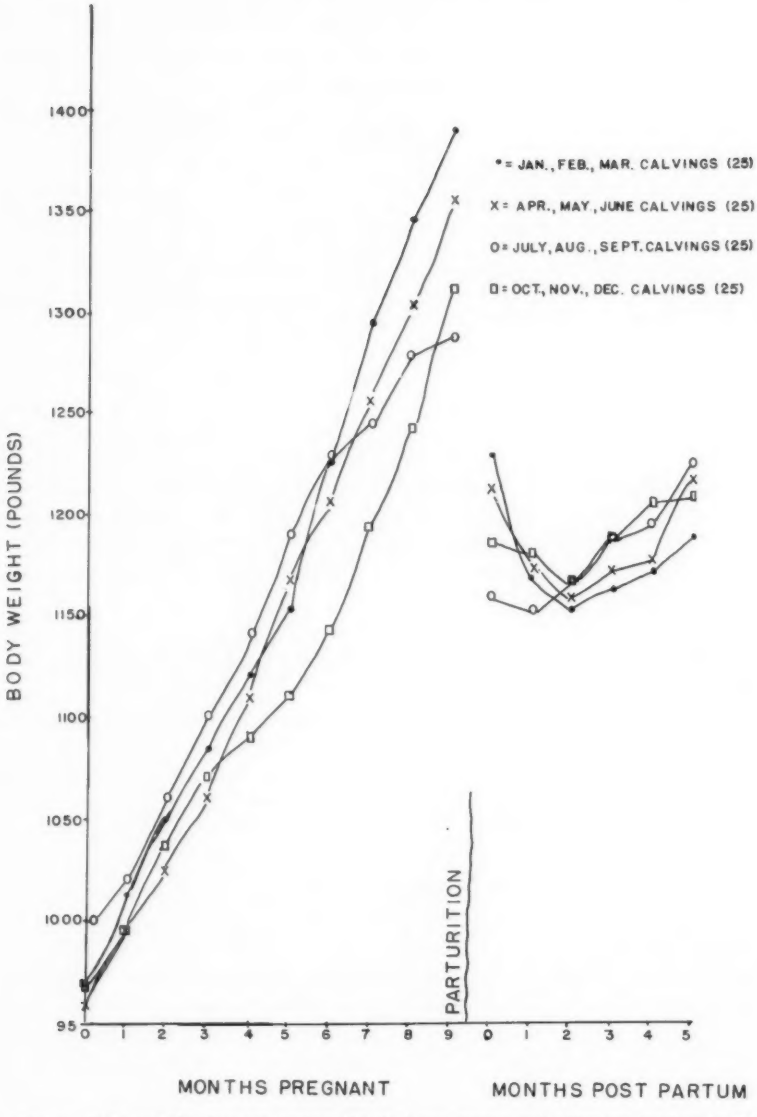


FIG. 1. Body weight changes during the first pregnancy and after parturition in Holstein heifers calving at different seasons of the year.

TABLE 1

Effect of season of calving on body weight changes during pregnancy in primiparous Holstein heifers

(25 heifers in each season group)

	Season of calving			
	January February March	April May June	July August September	October November December
	(lb.)			
Body weight at breeding	1,000	960	1,005	965
Body weight at end of pregnancy	1,385	1,356	1,290	1,320
Total gain	+385	+396	+285	+355
Body weight after calving	1,225	1,215	1,160	1,187
Loss at calving	−160	−141	−130	−133
Calf weight	86	85	82	85
Loss exclusive of calf	74	56	48	48
Gain during pregnancy exclusive of loss at parturition	225	255	155	222
Body weight at 5th mo. post-partum	1,195	1,212	1,215	1,208
Gain (breeding to 5th mo. post-partum)	+195	+252	+210	+243

ence to Table 1, the data from these 100 cows show that greatest total gain during pregnancy was made by heifers calving in the April, May, and June season, and the least total gain by those calving in the July, August, and September season. The difference in gains between season of calving is highly significant. Noticeable is the definite reduction in weight gain during the summer months in each of the weight-gain curves in Figure 1. However, the greatest reduction in total gain during pregnancy was in the group where the hot weather occurred towards the end of pregnancy. The loss in weight at parturition appeared to be greatest in the group calving during January, February, and March. The net gain during pregnancy (total gain-loss at calving) was greatest for the group calving in April, May, and June, and the least net gain was again those calving in July, August, and September. Howwever, by the 5th mo. post-partum, the body weights of all groups were similar, with the possible exception of the group calving in January, February, and March. This latter group was somewhat lighter in body weight than the other groups, probably because the 4th and 5th mo. post-partum were during June and July, when the depressing effect of hot weather may have again retarded gain.

In the analysis where the ten heifers of highest and ten of the lowest body weight at breeding time in each of the four seasons were grouped, the groups with the lowest body weights at breeding (average 960 lb.) gained 375 lb. during pregnancy, whereas the groups with the higher body weight at breeding time (1,052 lb.) gained 344 lb. during pregnancy. In the statistical analysis of these data, the season × month interaction was significant, but the group × season × month was not. This indicates that the weight changes during pregnancy were different for the heifers calving at different seasons of the year, but that weight at breeding did not influence the seasonal effect.

Shown in Table 2 is the pertinent portion of variance of these data after variance due to season, month of pregnancy, and individual was removed.

TABLE 2

Analysis of variance of body weights in primiparous heifers calving at different seasons

Source of variance	Degrees of freedom	Mean square
Season × monthly weight	27	10,667**
Weight group × season × month	27	878
Error	648	1,371

** Highly significant.

Body weight is important in milk yield of dairy cattle (1, and others), other factors being similar. A management factor that retards bodily development, such as calving during the summer appears to do, might be considered to be detrimental to the subsequent milk yield of the cow because of the effect on body weight. However, when the body weight of heifers freshening in the summer is observed several months post-partum (Figure 1), it can be seen that the suppression in rate of gain during the summer has been alleviated in the following fall months, so that the net effect of calving-season on body weight is unimportant. Apparently, the impetus to attain a definite mature size is only temporarily suppressed during the summer.

One may wonder whether alleviation of the summer suppression of rate of gain in heifers that calve in October, November, and December not only brings about accelerated rate of gain towards the end of their pregnancy but also a higher subsequent lactational yield. Increased release of growth hormone may be involved in both situations.

In the limited data available, it is noted that there is a significant correlation (44 comparisons: $r = +.40$) between body weight gain during the last 2 mo. of pregnancy and subsequent lactational performance, regardless of season of calving.

Frick *et al.* (3) show that in Connecticut, calvings in January, February, and March, as well as those in the fall months, are associated with above-average milk yield. In our data (Figure 1), rate of gain in the latter part of pregnancy is high for those heifers calving in January, February, and March, in April, May, and June, and in the fall months and, thus, Frick's data might lend indirect support to our observation of the significant positive correlation between rate of gain during the latter part of pregnancy and subsequent lactational performance. More specific and detailed study is needed to substantiate the relationship between gain during late pregnancy and lactational performance and the involvement of the growth hormone.

REFERENCES

(1) Brody, S. Bioenergetics and Growth. Reinhold Publishing Corp., New York. 1945.

(2) Cannon, C. Y., and Hansen, E. N. Freshening Date Changes of Cows in Iowa D.H.I.A. J. Animal Sci., 2: 112. 1943.

(3) Frick, George E., Mann, A. I., and Johnson, Stewart. The Relation of Season of Freshening to Milk Production. J. Dairy Sci., 30: 631. 1947.

(4) Morgan, R. F., and Davis, H. P. The Effect of Pregnancy and Parturition on the Weight of Dairy Cows. Nebraska Agr. Expt. Sta., Research Bull. No. 82. 1936.

(5) Putnam, D. N., and Henderson, H. O. The Effect of Pregnancy on the Body Weight of Dairy Cows. J. Dairy Sci., 29: 657. 1946.

GROWTH OF THE CALF UDDER USING DNA AS AN INDEX [1, 2]

RALPH WILLIAMS AND C. W. TURNER
Department of Dairy Husbandry, University of Missouri, Columbia

SUMMARY

Nine dairy calves were administered subcutaneously 30 μg. estradiol benzoate (EB) and 30 mg. progesterone (P) daily, starting at 2 mo. of age. Each month, the EB and P were increased 10 μg. and 10 mg., respectively. Three calves were sacrificed after 2, 4, and 6 mo. of treatment. The udders were removed and the glands in each quarter were dissected free of the surrounding fatty tissue, cut up into tiny pieces, and the fat and water extracted. The dry, fat-free tissue was then analyzed for the content of DNA per milligram. The total DNA in each quarter of the calf's udder was quite similar, but considerable variability was observed in either total DNA per udder or per 100 lb. body weight. In comparing the group injected for 4 mo. with the 2-mo. group, a marked increase in growth (DNA) was observed. It is believed that the DNA method will serve as a satisfactory method of quantitative estimation of normal and experimental gland growth in calves.

The normal and experimental growth of the mammary glands of mice (1, 2) and rats (4-8), using desoxyribonucleic acid (DNA) as an index, has been studied extensively in this laboratory.

In addition, the influence of estradiol benzoate and progesterone in stimulating growth of the udders of heifers has been reported. However, udder growth was measured indirectly by the yield of milk stimulated subsequently by estradiol benzoate (12, 13).

The present study was initiated to determine the possibility of using DNA as an index of quarter udder growth in calves when stimulated by estradiol benzoate and progesterone for variable lengths of time as a direct estimation of gland growth. Only very little study has been devoted to the problem of hormonal stimulation of udder growth in dairy calves (9).

MATERIALS AND METHODS

Nine dairy calves were administered subcutaneously 30 μg. estradiol benzoate (EB) and 30 mg. progesterone (P) daily, starting at 2 mo. of age. Each month the EB was increased 10 μg. and the P was increased 10 mg. Three calves were sacrificed after 2 mo., three after 4 mo., and three after 6 mo. of hormone treatment.

The udders were removed and halved. The glands in each quarter were carefully dissected free of the fatty pad in which they were growing. The peripheral zone of gland growth could be differentiated from the fat. Each

Received for publication April 14, 1961.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 2285. Approved by the Director.

[2] Aided in part by a grant from the U. S. Atomic Energy Commission [Contract No. AT (11-1)1301]. Syntex Animal Products Co. has generously contributed ovarian hormones.

quarter was placed in the deep freezer, after being cut into tiny pieces in preparation for fat extraction. Processing of the udder tissue from each quarter was conducted as previously described for the mammary gland of the rat (7).

RESULTS

It should be pointed out that these calves were subjected to ovarian hormone stimulation beginning at 2 mo. of age and continuing for 2, 4, and 6 mo. Since no control calves sacrificed at comparable ages are available, the growth-stimulating effect of the hormones can be observed only in relation to duration of the treatment.

It will be noted that the group injected for 2 mo. shows great variability in total DFFT, total DNA, and DNA/100 lb. body weight. It is not possible to say definitely whether this variability is due primarily to differences in the amount of gland present at the start of the experiment or to the rate of gland growth stimulated by the hormones. The fact that the DNA/mg DFFT is highest in the quarters of the calf showing the greatest total DNA is indicative of rapid growth (more cells per unit of gland). It has been shown in rats and mice that the DNA/mg DFFT is higher during periods of rapid growth (3). If this is true of calves, also, the data would indicate that the mammary glands of Calf No. 1 were being stimulated to more rapid growth than those of Calf No. 2 or No. 3.

The group subjected to 4 mo. of hormone treatment showed much greater uniformity in total DNA and per 100 lb. B.W. Udder growth was very rapid during this period, exceeding the 2-mo. group, in total DNA by 1,176.7 mg. and per 100 lb. B.W. by 332.4 mg.

The group of calves receiving the ovarian hormones for 6 mo. again showed great variability in response. One calf, No. 7, showed almost twice as much total DNA as any in the 4-mo. group, but the two other calves showed considerably less response, being in the range of those in the 4-mo. group or less.

While great increase in total DNA/100 lb. B.W. was observed in the group injected for 4 mo., in comparison with the 2-mo. group, little additional growth was observed in the group injected for 6 mo., although one calf showed greater growth.

Normal cows secrete more milk in the rear quarters than in the fore quarters (10). In the present study, the total DNA/100 lb. B.W. showed 24.4% in right front, 23.8% in left front, 24.7% in right rear, and 27.1% in left rear. The two halves were almost equal in total DNA, right half 49.1% and left half 50.9%.

DISCUSSION

The productivity of dairy cattle is, in part, dependent upon the number of milk-secreting cells in the udder and, in part, upon the intensity of milk secretion and its maintenance. In the rat and mouse, great variation has been observed in the total amount of gland tissue present at the end of pregnancy (2, 5), as well as when the glands were stimulated experimentally for an equal period (1, 7). It would be expected that similar great variability in total gland

TABLE 1

Mammary gland growth in calves measured by DNA

Herd No.	Body weight	Hormone injection period	DFFT/[a] quarter	DNA/mg DFFT/ quarter	Total DNA/ quarter	DNA/quarter/ 100 lb.
			(gm.)	*(μg.)*	*(mg.)*	*(mg.)*
1	224	2 mo.	1.32 ± .035	67.01 ± 2.15	88.19 ± 4.72	39.37 ± 2.11
2	216	2 mo.	0.48 ± .039	52.16 ± 4.12	25.10 ± 2.89	11.62 ± 1.33
3	208	2 mo.	0.35 ± .043	47.36 ± 4.58	16.28 ± 1.19	7.83 ± 0.97
Group mean/ quarter			0.71 ± .429	55.5 ± 8.36	43.2 ± 32.04	19.6 ± 14.06
4	320	4 mo.	6.25 ± .90	50.3 ± 2.09	313.6 ± 14.87	98.00 ± 4.77
5	360	4 mo.	6.53 ± .09	54.7 ± 4.75	357.4 ± 41.06	99.28 ± 11.41
6	307	4 mo.	6.55 ± .77	52.1 ± 1.32	341.1 ± 38.57	111.11 ± 12.55
Group mean/ quarter			6.4 ± .14	52.4 ± 1.80	337.4 ± 18.08	102.8 ± 5.90
7	421	6 mo.	10.63 ± 2.43	54.83 ± 3.60	587.6 ± 157.09	138.56 ± 37.29
8	407	6 mo.	10.85 ± 2.90	44.86 ± 3.46	437.9 ± 176.42	107.59 ± 25.52
9	396	6 mo.	4.8 ± 2.19	55.35 ± 8.26	267.6 ± 182.98	67.58 ± 14.61
Group mean/ quarter			8.8 ± 2.8	51.7 ± 4.83	431.0 ± 131.12	104.9 ± 29.42

[a] DFFT = dry, fat-free tissue.

growth exists in heifers at the end of pregnancy and, thus, accounts for part of the variation in milk production observed.

In rats and mice, DNA has been shown to be an excellent quantitative index of mammary gland growth. It is based upon the concept that all gland cells contain an equal amount of this nucleic acid (DNA) and, upon cell division, the DNA is doubled, i.e., each daughter cell contains the same amount as the parent cell. Thus, the increase in total DNA in the gland is believed to indicate the extent of cell multiplication (growth).

This study was initiated, primarily, to determine whether it would be feasible to attempt to use the DNA index in a study of normal and experimental growth of the udder of calves. Since the number of calves available was limited, all calves were treated with ovarian hormones for increasing time intervals.

The preliminary results to date clearly indicate that the DNA method can be used successfully with calves. The mammary gland in each quarter can be dissected free of the surrounding fatty tissue. The gland can be extracted and the dry fat-free tissue determined. In larger glands, it may be necessary to develop modifications of the fat and water extraction procedure.

Until data are available on the extent of DNA/100 lb. body weight in growing control animals, it will not be possible to evaluate the extent of growth stimulated by the ovarian hormones. Morphological study has indicated that little growth of the duct system occurs up to the time of sexual maturity (11). It would be expected that even 8-mo.-old calves would have relatively small glands as measured by DNA. If this is found to be true, then considerable growth of the gland has been induced by the hormone treatment.

It is interesting to observe that the four glands in each udder are quite similar in the extent of DNA, indicating a close approach to equality at this time; whereas, in the case of lactating animals, the rear halves frequently produce 60% in contrast to 40% produced by the fore halves (10). In none of these calves had the glands developed into the full fatty tissue pad available. It is possible that the growth of the fore quarters becomes restricted only as the fatty pad is filled with gland tissue. In other words, the potential for growth is the same in all quarters, but may become reduced by space limitations of the udder quarters.

It is evident from this preliminary study that great variability exists in the response of calves to ovarian hormones. Large groups of calves will be required in each lot to overcome this variability. Two alternatives are suggested: One would involve the use of identical twin calves; the other, to remove one-half of the udder as a control, since the two halves are quite similar in total DNA.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mary E. Powell.

REFERENCES

(1) Anderson, R. R., Brookreson, A. D., and Turner, C. W. Experimental Growth of Mammary Gland in Male and Female Mice. Proc. Soc. Exptl. Biol. Med., 106: 567. 1961.

(2) Brookreson, A. D., and Turner, C. W. Normal Growth of Mammary Gland in Pregnant and Lactating Mice. Proc. Soc. Exptl. Biol. Med., 102: 744. 1959.

(3) Damm, H. C., and Turner, C. W. Desoxyribosenucleic Acid as Index of Mammary Gland Growth of Mice. Proc. Soc. Exptl. Biol. Med., 95: 466. 1957.

(4) Griffith, D. R., and Turner, C. W. Normal Growth of Rat Mammary Glands During Pregnancy and Lactation. Proc. Soc. Exptl. Biol. Med., 102: 619. 1959.

(5) Griffith, D. R., and Turner, C. W. Normal Growth of Rat Mammary Glands During Pregnancy and Early Lactation. Proc. Soc. Exptl. Biol. Med., 106: 448. 1961.

(6) Griffith, D. R., and Turner, C. W. Thyroxine and Mammary Gland Growth in Rat. Proc. Soc. Exptl. Biol. Med., 106: 873. 1961.

(7) Moon, R. C., Griffith, D. R., and Turner, C. W. Normal and Experimental Growth of Rat Mammary Gland. Proc. Soc. Exptl. Biol. Med., 101: 788. 1959.

(8) Moon, R. C., and Turner, C. W. Thyroid Hormone and Mammary Gland Growth in the Rat. Proc. Soc. Exptl. Biol. Med., 103: 149. 1960.

(9) Sykes, J. F., and Wrenn, T. R. Hormonal Development of the Mammary Gland of Dairy Heifers. J. Dairy Sci., 33: 194. 1950.

(10) Turner, C. W. The Functional Individuality of the Mammary Glands of the Udder of the Dairy Cow. Missouri Research Bull. 211. 1934.

(11) Turner, C. W. The Mammary Gland. Lucas Brothers Publishers, Columbia, Missouri. 1952.

(12) Turner, C. W. The Experimental Induction of Growth of the Cow's Udder and the Initiation of Milk Secretion. Missouri Research Bull. 697. 1959.

(13) Williams, Ralph, and Turner, C. W. Effect of Increased Levels of Ovarian Hormones and Duration of Treatment on the Experimental Induction of Growth of the Cow's Udder. J. Dairy Sci., 44: 524. 1961.

UDDER CAPACITY ESTIMATES AS AFFECTED BY RATE OF MILK SECRETION AND INTRAMAMMARY PRESSURE[1]

H. A. TUCKER,[2] R. P. REECE, AND R. E. MATHER
Department of Dairy Science
New Jersey Agricultural Experiment Station, New Brunswick

SUMMARY

An extra-period Latin-square design balanced for sequence effects, with four groups of five Jerseys each, was used to study udder capacity. A group of five Brown Swiss provided aditional information. Experimental intervals were 8, 12, 16, 20, and 24 hr. in length. The intervals prior to the 8-, 12-, 16-, and 20-hr. experimental intervals were 6, 2, 8, and 4 hr., respectively, and thus too short to affect the experimental results. The 24-hr. interval had preceding intervals of 8, 12, 14, 16, 20, and 24 hr., but they produced no significant effect on total pounds of milk. Sequence effects were not significant. Oxytocin (20 I.U.) was injected before and after each experimental interval to eliminate residual milk effects. From these data, estimates of udder-holding capacity and capacity to secrete against the intramammary pressure and/or the toxin-like action of accumulated milk were obtained.

With increasing milking intervals the total pounds of milk increased asymptotically for all groups. The estimated theoretical udder capacities for the five groups of animals in this study were 42.4, 49.9, 55.8, 51.3, and 63.2 lb. of milk. Thirty-five hours was the estimated length of time before milk secretion approached zero. Increases in total milk caused increases in intramammary pressure. With increases in milking interval, total milk, and/or intramammary pressure, both the average and instantaneous secretion rates declined.

Several methods have been suggested for measuring maximum udder capacity. Matthews *et al.* (7) measured udder capacity in terms of the amount of a formalin solution, on a milk-equivalent basis, which could be infused and held within an excised udder. Turner (13) indicated that an end point for filling the udder under various milking routines was difficult to fix, but that the best estimate for maximum udder capacity was based on milk yield plotted against time.

The results of other workers (1, 2, 5, 9) have indicated that milk secretion rates declined with increasing milking intervals. Subsequent research by Turner (12-15) and Elliott and Brumby (4) suggested that at least 16 to 20 hr. had to transpire before secretion rates declined. In addition, these latter experiments pointed out two sources of error, residual milk and long preceding intervals, which were not accounted for in earlier experiments. The more recent work of Schmidt (10), in an experiment designed to account for the effects of residual milk and long preceding intervals, has again suggested that milk secretion rates decline with increasing milking intervals after 12 hr.

Received for publication February 2, 1961.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University, Department of Dairy Science, New Brunswick.

[2] These data are from a thesis presented by the senior author to the graduate faculty of Rutgers—The State University in partial fulfillment of the requirements for the M.Sc. degree.

In this present study the amount of milk secreted and stored for a 24-hr. period was used as an estimate of udder capacity. Such a figure does not represent maximum udder capacity; however, it does represent significant udder capacity, inasmuch as milking intervals greater than 24 hr. are of no practical importance. In addition, since the literature shows contradictory results regarding the influence of the milking interval on milk secretion rate, it was thought desirable to reinvestigate this problem.

EXPERIMENTAL PROCEDURE

Twenty purebred Jersey and five purebred Brown Swiss cows were used in this experiment. To maintain conditions during the experiment as nearly normal as possible, the cows after freshening were returned to their usual stalls in the dairy barn. Two to four weeks usually elapsed between calving and the start of an experiment for any cow. No experiment was started until the udder was free from edema, the cow was on full feed, and the production had leveled off; the peak of lactation was determined by inspection of daily milk weights.

The 20 Jerseys were divided into four groups similar in number of lactations, A, B, C, and D averaging 1.0, 2.8, 5.4, and 7.8 lactations, respectively. The five Brown Swiss, Group E, averaged 4.0 lactations and provided additional information. The experimental intervals were 8, 12, 16, 20, and 24 hr. An extra-period Latin-square design, balanced for carry-over effects, as outlined by Lucas (6), was used. Five animals, five experimental treatments (milking intervals), and six periods comprised a square. The setup for the Jerseys is given in Table 1. The table shows that Cow a started at the 8-hr. interval. The group of Brown Swiss had the same sequence as Group B. Since it was desired to have all the experimental intervals end at the morning milking, in an effort to minimize milking schedule disturbances, the time of milking at the beginning

TABLE 1

Milking interval sequence for 20 Jersey cows

	Group A					Group B				
Animals	a	b	c	d	e	f	g	h	i	j
Periods [a]										
1	8	12	16	20	24	8	12	16	20	24
2	12	20	8	24	16	24	16	8	12	20
3	16	8	24	12	20	12	24	20	8	16
4	20	24	12	16	8	20	8	24	16	12
5	24	16	20	8	12	16	20	12	24	8
6	24	16	20	8	12	16	20	12	24	8

	Group C					Group D				
Animals	k	l	m	n	o	p	q	r	s	t
Periods [a]										
1	8	12	16	20	24	8	12	16	20	24
2	16	8	24	12	20	12	24	20	8	16
3	12	20	8	24	16	16	20	12	24	8
4	24	16	20	8	12	20	8	24	16	12
5	20	24	12	16	8	24	16	8	12	20
6	20	24	12	16	8	24	16	8	12	20

[a] Intervals for these periods (8, 12, 16, etc.) are given in hours.

of the experimental interval was varied. Thus, the length of the interval from the last regular milking to the beginning of the experimental intervals also varied. To keep this preceding interval as short as possible, a regular milking was carried out at the normal milking time previous to the first experimental milking of the interval. The purpose of this was to minimize any preceding-interval effect on the experimental interval. A possible disadvantage of this additional milking was that it might have brought about greater hormonal stimulation and, thereby, increased milk secretion. For the shorter intervals (i.e., 8 and 12 hr.) there were three milkings in 24 hr.; for the 16- and 20-hr. intervals there were two milkings in 24 hr.; and for the 24-hr. interval there was, of course, only one milking in 24 hr.

To eliminate residual milk effects 20 I.U. oxytocin (Pitocin-Parke, Davis & Co.) was injected into the subcutaneous abdominal vein at the beginning and end of each experimental interval.

Intramammary pressures were measured with a sphygmomanometer at the end of each experimental interval, before stimulation of the milk-ejection reflex. The sphygmomanometer was attached by rubber tubing to a teat cannula, which was inserted into the right-fore teat. The sphygmomanometer was held at a standard 18 in. above the floor of the cow's udder.

The amount of milk removed from the udder after a 24-hr. period was used as an estimate of udder capacity. Under the conditions of this experiment this takes into account both the capacity to secrete and the capacity to hold milk. Since the udder is an elastic gland, the holding capacity changes as milk accumulates. The maximum holding capacity is not reached until the udder is stretched to its physiological limit.

RESULTS AND DISCUSSION

Differences among the carry-over effects of the preceding experimental intervals for the Jerseys, independent of the effects of the immediate preceding interval, were not significant.

Since Elliott (3) indicated that a preceding interval had to be at least 12 hr. in length to affect significantly the subsequent interval, intervals prior to the 8-, 12-, 16-, and 20-hr. experimental intervals (6, 2, 8, and 4 hr., respectively) were too short to affect the experimental results. The 24-hr. interval, on the other hand, had preceding time periods of 8, 12, 14, 16, 20, or 24 hr., depending upon its position in the sequence. By an analysis of covariance, as outlined by Snedecor (11), the milk production in 24 hr. was analyzed for differences due to the preceding interval, with adjustment for most of the differences due to the cow's characteristics by using her average level of production in experimental intervals other than the 24-hr. interval. These adjusted means did not differ significantly, and did not show any apparent trend. Thus, any carry-over effect of the experimental interval per se, the preceding-interval effect, and the residual milk effect were either accounted for or eliminated.

The relationship between milking interval and total pounds of milk secreted was not linear (Figure 1). Although the total pounds of milk increased, the

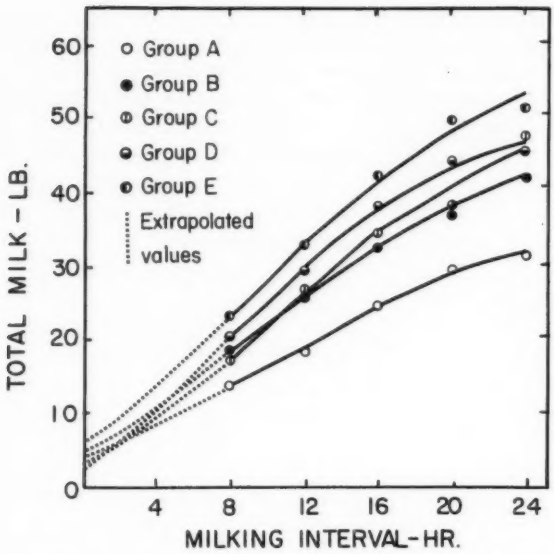


FIG. 1. Asymptotic regression curves of total pounds of milk (M) on milking interval (H) for five groups of cows.

Group A; $\log M = 1.627 - 0.495\ (0.702^x)$
Group B; $\log M = 1.698 - 0.435\ (0.650^x)$
Group C; $\log M = 1.746 - 0.503\ (0.652^x)$
Group D; $\log M = 1.710 - 0.407\ (0.579^x)$
Group E; $\log M = 1.801 - 0.436\ (0.651^x)$

$$x = \frac{H - 8}{4}$$

rate of increase decreased with increasing milking intervals. Theoretically, the total pounds of milk would approach a maximum. The curve describing this relationship would thus be asymptotic and these curves were calculated by the method of Patterson (8). The use of the common logarithm of the total milk seemed to result in a more nearly correct fit of the curve to the observed points than did total pounds of milk per se. The predicted logs of the total pounds of milk were then reconverted to the arithmetic scale (i.e., total pounds of milk). These reconverted values and the actual averages for each group were plotted (Figure 1). The estimated 4-hr. and 0-hr. total pounds of milk also were calculated. The extrapolated values at 4 hr. appeared reasonably close to anticipated values. At the 0 hr., however, the values were much higher than expected. The explanation is that the curve based on logarithms is also asymptotic to the base line and could, therefore, not be 0 milk at 0 hr.

Provided additional adverse factors did not enter the milk secretion process, after 24 hr., the average maximum total pounds of milk which theoretically

could have been secreted were about 42.4, 49.9, 55.8, 51.3, and 63.2 for Groups A through E, respectively.

The average milk secretion rates declined with increasing milking intervals (Table 2). Average secretion rates represent the average of all the secretion rates up to a given time. Thus, such an average does not represent the true secretion rates at any given time, as does the instantaneous secretion rate. First derivatives of the equations for the log total pounds of milk asymptotic curves gave these instantaneous rates (Figure 2). As expected, the instantaneous rates declined to a much greater extent with increasing time than did the average secretion rates.

TABLE 2

Average secretion rate in pounds of milk per hour

Animal groups	8 hr.	12 hr.	16 hr.	20 hr.	24 hr.
			(lb/hr)		
A [a]	1.70	1.55	1.54	1.46	1.31
B [b]	2.29	2.15	2.07	1.87	1.74
C [c]	2.18	2.25	2.12	1.90	1.98
D [d]	2.52	2.45	2.36	2.19	1.89
E [e]	2.91	2.67	2.62	2.47	2.14
Average A, B, C, D	2.17	2.10	2.02	1.86	1.73
Average all groups	2.32	2.22	2.14	1.98	1.81

[a] Jerseys—first lactation.
[b] Jerseys—average 2.8 lactations.
[c] Jerseys—average 5.4 lactations.
[d] Jerseys—average 7.8 lactations.
[e] Brown Swiss—average 4.0 lactations.

The instantaneous secretion rates also provided a means of estimating the time required for the asymptotes of log total pounds to be approached closely. The total pounds of milk at the 24-hr. interval were subtracted from the theoretical maximum total pounds of milk (asymptotes). Dividing this value by the instantaneous rate at the 24-hr. interval resulted in a new value, which was added to 24 hr. to yield an estimate of the time required for almost maximum capacities to be attained. The estimated times required were 38, 34, 32, 33, and 36 hr. for Groups A through E, respectively. The over-all average time was 35 hr., in agreement with the results of Turner (13). To obtain these values, the instantaneous secretion rates per hour were assumed to remain at the same level as the 24-hr. instantaneous rate, which would make these values lower than they should be.

The graphic relationships of total milk and intramammary pressure (Figure 3), total milk and instantaneous secretion rate (Figure 3), and instantaneous secretion rate and intramammary pressure (Figure 4), suggest the close interrelationship among these in the over-all milk secretion process. Assuming that the number of alveolar cells remain constant, these variables may be assumed to be interrelated in the following way: Immediately after milking, or at least shortly after milking, the stimulus for secretion would be at its greatest, and in

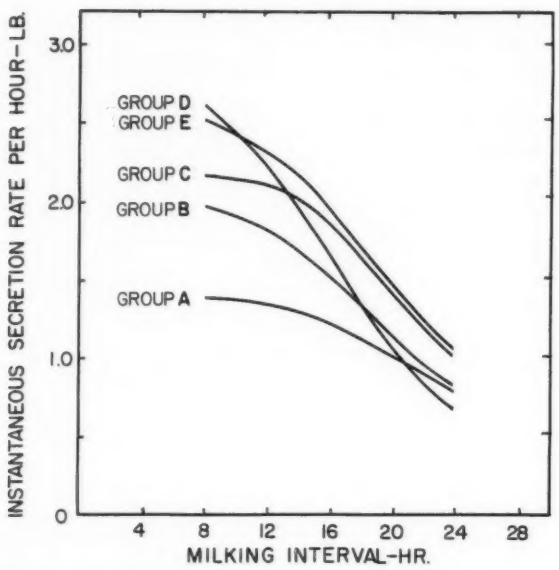


FIG. 2. Change of instantaneous secretion rate per hour (I) with milking interval (H) for five groups of cows.

$$\text{Group A; } I = 0.25\,(-0.495)\,(0.702^{x})\,(ln\ 0.702)\left(\frac{M}{0.4343}\right)$$

$$\text{Group B; } I = 0.25\,(-0.435)\,(0.650^{x})\,(ln\ 0.650)\left(\frac{M}{0.4343}\right)$$

$$\text{Group C; } I = 0.25\,(-0.503)\,(0.652^{x})\,(ln\ 0.652)\left(\frac{M}{0.4343}\right)$$

$$\text{Group D; } I = 0.25\,(-0.407)\,(0.579^{x})\,(ln\ 0.579)\left(\frac{M}{0.4343}\right)$$

$$\text{Group E; } I = 0.25\,(-0.436)\,(0.651^{x})\,(ln\ 0.651)\left(\frac{M}{0.4343}\right)$$

$$x = \frac{H-8}{4} \qquad M = \text{Total pounds of milk}$$

the absence of inhibitory effects the rate of secretion would be highest for any given cow. As the milk accumulates in the alveoli, the intramammary pressure begins to increase. At the lower levels of this intramammary pressure the inhibitory effect on the secretion rate is negligible. The increasing intramammary pressure is caused by the increased total pounds of milk and the reduced amount of maximum udder volume still available for storing the synthesized milk. As the amount of milk accumulation begins to approach that point where the udder is being stretched, the intramammary pressure increases at a greater rate. This may be seen in Figure 3 between 34-40 total pounds of milk. As a result of the increasing pressure, secretion of milk is depressed, due either to the effects of

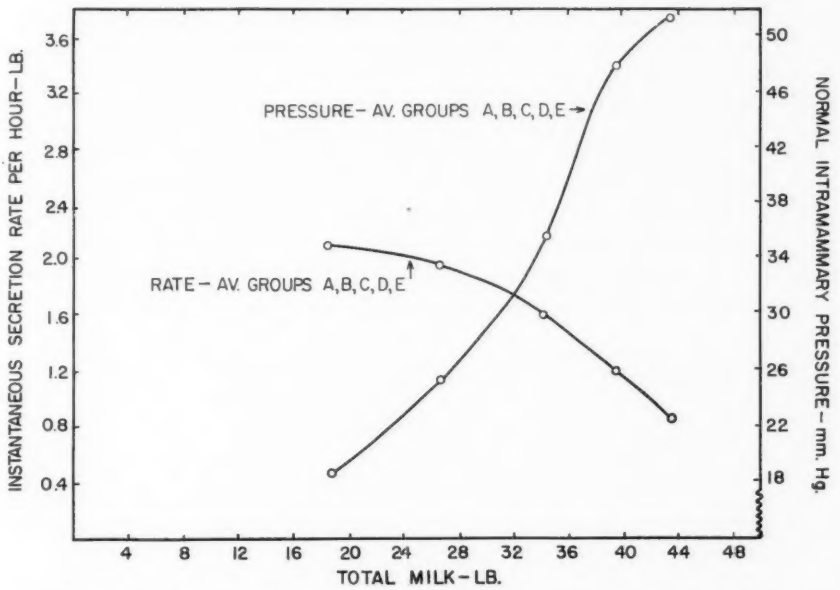


FIG. 3. Relation between total pounds of milk and normal intramammary pressure and total pounds of milk and instantaneous secretion rate.

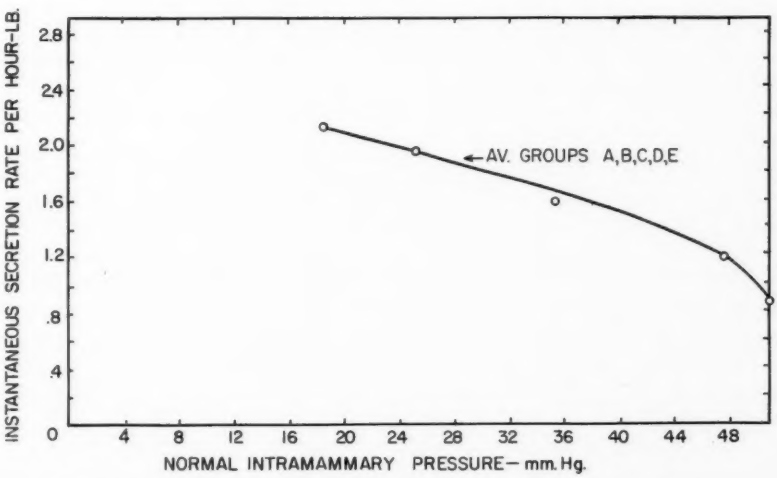


FIG. 4. Relation between instantaneous secretion rate per hour and normal intramammary pressure.

the pressure itself and the inability of the cells to secrete against this pressure, or to a possible chemical inhibition of secretion. It is also possible that there is resorption of some components of the milk, including water, which would make it seem that secretion rate had been depressed more than it actually was. In turn, the reduced secretion rate causes less accumulation of milk and less increase in intramammary pressure in the next time period.

No attempt was made to determine the relative importance of intramammary pressure and total milk on the secretion rate, since intramammary pressure would be present only if milk were present in the udder. Thus, for all practical purposes the depressing effects of these two variables on the secretion process were inseparable.

ACKNOWLEDGMENT

This study was supported by a grant from the Frelinghuysen Foundation.

REFERENCES

(1) Bartlett, S. Studies in Milk Secretion Based on the Variations and Yields of Milk and Butter Fat Produced at Morning and Evening Milkings. J. Agr. Sci., 19: 36. 1929.

(2) Edwards, J. Factors Influencing the Relationship Between the Secretion of Milk and Butterfat. J. Agr. Sci., 40: 100. 1950.

(3) Elliott, G. M. The Direct Effect of Milk Accumulation in the Udder of the Dairy Cow upon Milk Secretion Rate. Dairy Sci. Abstr., 21: 435. 1959.

(4) Elliott, G. M., and Brumby, P. J. Rate of Milk Secretion with Increasing Interval Between Milking. Nature, 176: 350. 1955.

(5) Johansson, I. Variations in the Fat Content of Milk Caused by Varying Degrees of Udder Evacuation During Milking. Proc. Intern. Dairy Congr. (Stockholm), 1: 171. 1949.

(6) Lucas, H. L. Extra-Period Latin-Square Change-Over Designs. J. Dairy Sci., 40: 225. 1957.

(7) Matthews, C. A., Swett, W. W., and Fohrman, M. H. Weight and Capacity of the Dairy Cow Udder in Relation to Producing Ability, Age, and Stage of Lactation. USDA, Tech. Bull. 989. 1949.

(8) Patterson, H. D. A Simple Method for Fitting an Asymptotic Regression Curve. Biometrics, 12: 323. 1956.

(9) Ragsdale, A. C., Turner, C. W., and Brody, S. The Rate of Milk Secretion as Affected by an Accumulation of Milk in the Mammary Gland. J. Dairy Sci., 7: 249. 1924.

(10) Schmidt, G. H. Effect of Milking Intervals on the Rate of Milk and Fat Secretion. J. Dairy Sci., 43: 213. 1960.

(11) Snedecor, G. W. Statistical Methods. 5th ed. The Iowa State College Press, Ames. 1956.

(12) Turner, H. G. Dependence on Residual Milk in the Udder of the Cow upon Total Yield: Its Bearing upon Supposed Inhibition of Secretion. Australian J. Agr. Research, 4: 118. 1953.

(13) Turner, H. G. Changes in Capacity of the Udder of the Dairy Cow During the Course of Lactation. Australian J. Agr. Research, 6: 145. 1955.

(14) Turner, H. G. Sources of Variation in Residual Milk and Fat in Dairy Cows: Their Relation to Secretion Rates and Persistency of Lactation. Australian J. Agr. Research, 6: 514. 1955.

(15) Turner, H. G. The Effect of Unequal Intervals Between Milkings upon Milk Production and Diurnal Variation in Milk Secretion. Australian J. Agr. Research, 6: 530. 1955.

METHOD FOR INCREASED MILK PRODUCTION WITH ROTATIONAL GRAZING [1]

H. T. BRYANT, R. E. BLASER, R. C. HAMMES, JR., AND W. A. HARDISON [2]
Departments of Agronomy and Dairy Science
Virginia Agricultural Experiment Station, Blacksburg

SUMMARY

The effect of high and low selective grazing by dairy cows on milk production, changes in body weight, consumption, and digestibility of dry matter ingested, was studied for three grazing seasons. A system of rotational grazing was followed; one group of cows (top grazers) grazed about half and a second group (bottom grazers) the remainder of the grazeable herbage.

The milk production from cows grazing an alfalfa-orchardgrass mixture for the first 98 days in 1955, 126 days in 1956, and the last 70 days in 1957 was 39.5, 33.1, and 36.0 lb. for the top grazers, as compared to 30.7, 27.6, and 27.8 lb. for the bottom grazers, respectively. For the groups not fed shelled corn, the milk production of the top grazers was 25, 53, and 49% greater, respectively, than the bottom grazers for the above comparison periods.

During the first 49 days in 1957 that cows grazed Ladino clover–orchardgrass, the top grazers averaged 43.6 lb. of milk daily, as compared to 35.2 lb. for the bottom grazers. Top grazers not fed shelled corn produced 41% more milk daily than the bottom grazers not fed shelled corn.

Changes in body weight between the cows due to treatments were not statistically significant.

The data from the chemical analyses of the forages before and after top and bottom grazing indicate that the bottom grazers consumed an inferior quality forage as compared with top grazers. In 1956, the top grazers' average daily forage consumption was 33.4 lb. of dry matter (64.4% digestible), as compared to a consumption of 28.5 lb. of dry matter (61.8% digestible) by the bottom grazers.

Animals graze selectively, i.e., ingested herbage is higher in digestibility, fat, and protein, and lower in crude fiber, than the total available herbage as well as the ungrazed residue (2, 8, 13, 15, 16). Heavy stocking with concurrent low availability and low selectivity of herbage results in a lower output per animal than light stocking (1, 2, 5, 9-11). There is high selective grazing when animals are turned into a fresh rotationally grazed pasture and low selective grazing as the grazeable herbage is consumed. Because of rapid changes in degree of selectivity within a rotationally grazed pasture, there are large daily fluctuations in animal output (1, 2, 4, 16, 18).

The purpose of this study was to measure the effect of high and low selective grazing on milk production, changes in body weight, and consumption and digestibility of dry matter in lactating dairy cows. The determination of composition of the forage available to animals on the different grazing systems also was deemed important. High and low selectivity of forage was accomplished

Received for publication March 4, 1961.

[1] This work was partially supported by the Old Dominion Foundation.

[2] Present address: Southeastern Baptist Seminary, Wake Forest, North Carolina.

by allowing one group of cows to graze about half and a second group the remainder of the grazeable herbage. Those cows which were first introduced into a pasture are referred to as top grazers (high selective grazing) and those introduced later are bottom grazers (low selective grazing).

EXPERIMENTAL PROCEDURES

Eight lactating Holstein cows (divided equally into top and bottom grazers) were used in each of three grazing experiments during the 1955, 1956, and 1957 pasture seasons. Top grazers consumed about one-half of the grazeable herbage; the bottom grazers ate the remainder. Pastures were managed so that the grazing period was about two days for each group of cows. Such a grazing schedule was realized by adjusting the stocking rate with additional grazers (3). Two factorial treatments (ground shelled corn supplement vs. no supplement) were imposed within each of the two grazing groups. There were two replications in the field layout.

For the last one-third (49 days) of the 1955 grazing season, top and bottom grazers were shifted; however, the corn feeding schedule was not changed. During 1956 and 1957, animals were maintained on the same treatments during the entire grazing seasons. Pasture during the experiment was an alfalfa-orchardgrass mixture, with the exception of 49 days in 1957, when a Ladino clover–orchardgrass mixture was used. Total days cows were on pasture treatments during the 1955, 1956, and 1957 seasons were 147, 126, and 119, respectively.

Each year a pregrazing standardization period preceded the treatment period. The standardization period was 7 wk. in 1956 and 4 wk. in 1955 and 1957. During standardization, cows were fed alfalfa-orchardgrass hay or silage ad libitum, plus a concentrate mixture containing 20% total crude protein (641 lb. of ground shelled corn and 359 lb. of cottonseed meal). The feeding rate of the concentrate was 1 lb. to each 4 lb. of milk in 1955 and 1956 and 1:6 in 1957.

Based on the standardization period, cows were allotted to their respective treatments. An attempt was made to balance the grazing groups for milk production, stage of lactation, body weight, and age. The rate of feeding corn supplements during the pasture treatments was based on milk production during standardization and remained constant during each grazing season. One pound of ground shelled corn was fed to each 8 lb. of milk in 1955 and 1956 and 1:8 of 4% fat-corrected milk (FCM) in 1957. Hereafter, the shelled corn supplement will be termed supplement.

Milk weights were recorded twice daily and a one-day composite sample was obtained monthly for fat content (analyzed according to the Babcock procedure). Milk is reported as 4% fat-corrected milk (FCM). The cows were weighed weekly and for two consecutive days at the beginning and end of each pasture treatment.

Data on available and ungrazed herbage yields were obtained by clipping four random samples (4-ft. square) within a pasture as follows: (a) before

top grazing, (b) after top grazing, (c) after bottom grazing. Herbage samples were composited by replication for chemical analysis. Several pastures were sampled at random each year.

In 1956, during June, July, and September, estimates of herbage digestibility and dry matter intake were obtained for the nonsupplemented experimental top and bottom grazers by using the chromogen (17) and chromic oxide technique (7). Chromic oxide (10 g.) was administered and grab fecal samples were collected twice daily after milking.

The animal production data analyzed each year were adjusted for variation in milk production between cows during the standardization period. An index to the milk-stimulating effect of the pasture on each cow's milk production was determined by computing a persistency rating based on the ratio between each week's and the prior week's production.

EXPERIMENTAL RESULTS

In 1955, the average daily milk production of top grazers was 39.5 lb., as compared with 30.7 lb. for bottom grazers during the 98-day period, Figure 1.

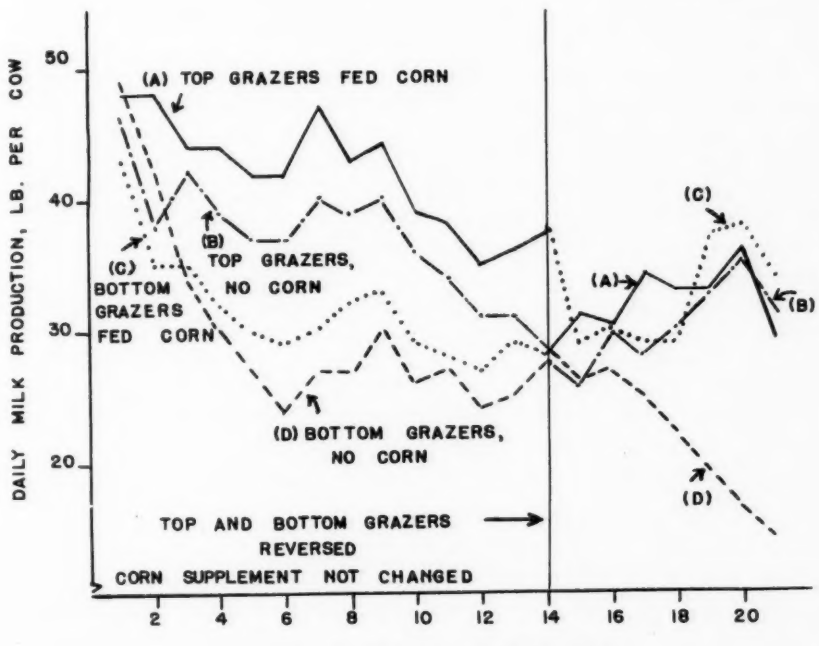


FIG. 1. Average daily production of 4% fat-corrected milk by weeks of top- and bottom-grazing cows, with and without feeding shelled corn, on rotationally grazed alfalfa-orchardgrass. Milk production differed significantly at the 1% level before and after reversal for grazing treatments. 1955.

When the supplement was not fed, the top grazers produced 24% more milk than the bottom grazers. The shifting of the top grazers to bottom grazers for 49 days at the end of the season reduced the average daily milk production by 12.7 lb.; conversely, there was an increase of 0.5 lb. when bottom grazers were switched to top grazers. The top grazers' mean weekly milk production persistency before the reversal was 98.3%, as compared to 94.8% after the top grazers became bottom grazers. The bottom grazers' milk persistency before the reversal was 96.6%, as compared to 101.6% after the cows were shifted to top growth. The shifting of the nonsupplemented top and bottom grazers resulted in even greater differences in milk production, Figure 1. Conversely, the shifting of bottom grazers to top grazers (without supplements) increased their production. After shifting the grazing treatments, supplements apparently caused greater stimulation of production for bottom than for top grazers, as shown by the significant interaction between supplements and grazing treatment.

During 1956, the average daily milk production for all cows was 33.1 lb. for the top and 27.6 lb. for the bottom grazers (Figure 2). The mean weekly persistency of milk production was 96.3 and 94.6%, for the top and bottom grazers, respectively. In the unsupplemented groups, the top grazers averaged 28.8 lb., as compared with 18.8 lb. for bottom grazers.

During the first 49 days of 1957 that cows grazed Ladino clover–orchardgrass, the top grazers averaged 43.6 lb. of milk daily, as compared to 35.2 lb. for the bottom grazers (Figure 3). The top grazers' and bottom grazers' mean weekly milk production persistency was 102.8 and 96.7%, respectively. Unsupplemented top grazers produced 12.4 lb. more milk daily than the unsupplemented bottom grazers. Increased production per pound of grain fed was again greater among the bottom than among the top grazers, as shown by the significant interaction.

The top grazers averaged 36.0 lb. of milk daily during the 70 days of 1957 that alfalfa-orchardgrass was grazed, as compared to 27.8 lb. for the bottom grazers. The mean weekly persistency of milk production was 98.9% for the top grazers, as compared to 95.9% for the bottom grazers. During this period, the unsupplemented top grazers produced 49% more milk than the unsupplemented bottom grazers.

Both the top and bottom grazers refused some supplement; refusals were largest during the spring flush growth. Each year corn supplement was fed at a constant rate (based upon production during standardization). The bottom grazers ate 0.025 lb. more supplement per pound of milk than top grazers.

Body weights of the supplemented top grazers underwent no appreciable change during the pasture periods, whereas unsupplemented top grazers lost an average of 0.27 lb. per day. Supplemented bottom grazers gained an average of 0.23 lb. daily, as compared to a loss of 0.12 lb. for the unsupplemented bottom grazers. However, these changes in body weight were not statistically significant during any of the three grazing seasons.

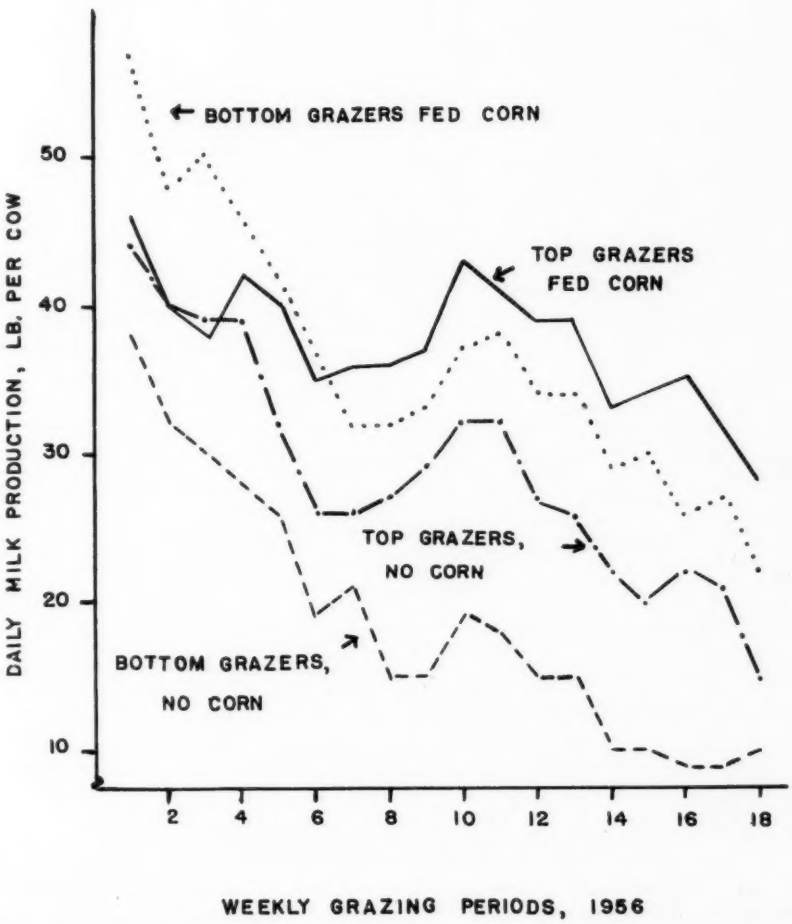


FIG. 2. Average daily production of 4% fat-corrected milk by weeks of top- and bottom-grazing cows, with and without feeding shelled corn, on rotationally grazed alfalfa-orchardgrass pastures. Milk production differed significantly at the 5% level for grazing treatments and supplements. 1956.

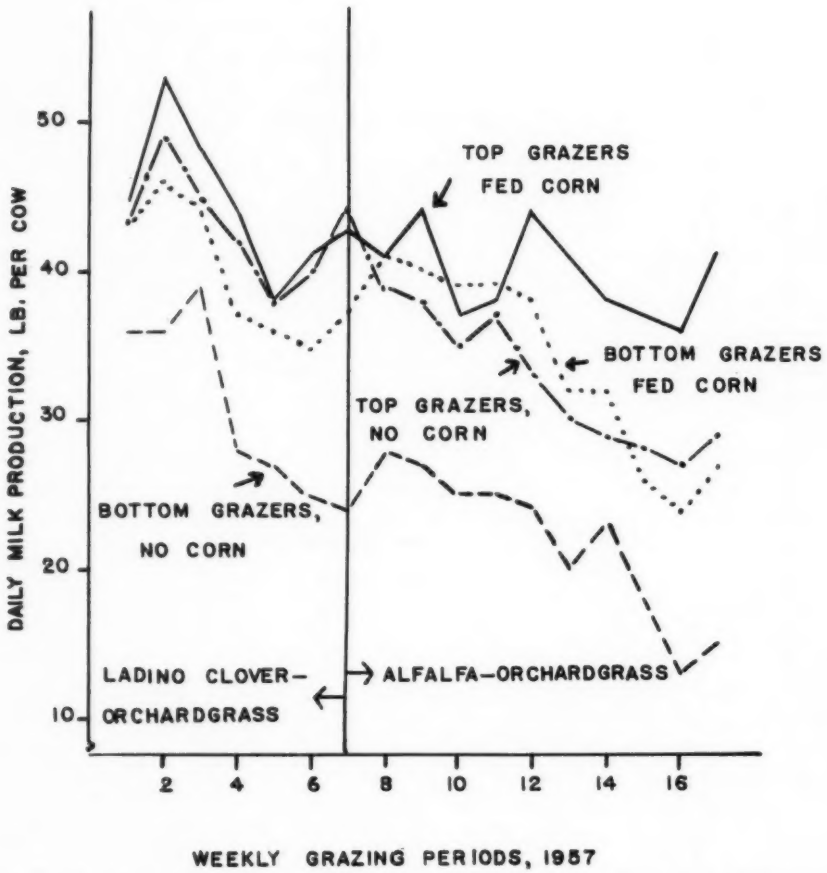


FIG. 3. Average daily production of 4% fat-corrected milk by weeks of top- and bottom-grazing cows, with and without feeding shelled corn, on two pasture mixtures. Milk production during period Ladino clover–orchardgrass was grazed differed significantly at the 1% level for grazing treatments and at the 5% level for supplements and the supplement by grazing treatment interaction. 1957.

Herbage composition before and after top and bottom grazing for the trials is given in Table 1. The forage remaining after each grazing treatment was less in crude protein, ether extract, nitrogen free extract, and ash, and greater in crude fiber, than that available at the beginning of the grazing treatments. Thus, it is evident that bottom grazers consumed an inferior quality of forage, as compared with top grazers.

TABLE 1

Average herbage composition (dry basis) for methods of grazing an alfalfa-orchardgrass mixture

Rotationally grazed herbage	Herbage composition *(%)*				
	Crude protein	Crude fiber	Ether extract	Nitrogen-free extract	Ash
Before top grazing	20.3	19.6	1.9	47.0	8.2
After top grazing, also before bottom grazing	14.6	27.1	1.6	46.0	7.7
After bottom grazing	12.2	30.2	1.4	45.7	7.5

The average dry matter digestibility of the forage ingested by the top and bottom grazers, as determined by the chromogen technique, was 64.4 and 61.8%, respectively (Table 2). The average daily dry matter intake, as determined by the chromic oxide technique, was 33.4 lb. for the top grazers and 28.5 lb. for the bottom grazers.

TABLE 2

Dry matter digestibility and daily herbage intake for dairy cows with top and bottom grazing on alfalfa-orchardgrass pastures, 1956

Grazing period	Grazing management			
	% Dry matter digestibility		Daily dry matter intake *(lb.)*	
	Top grazing	Bottom grazing	Top grazing	Bottom grazing
June	58.3	56.2	34.0	27.4
July	66.6	61.6	35.5	33.2
September	68.3	67.6	30.6	25.0
Average	64.4	61.8	33.4	28.5

DISCUSSION

The greater milk production of the top than for the bottom grazers can be attributed to a greater selectivity of forage and greater dry matter ingestion among top grazers. Top grazers usually grazed the upper half of the alfalfa plants which averaged 58% leaves (dry weight basis), as compared to 28% leaves for the lower half of the alfalfa plant. Intake of a better quality pasturage is also indicated by the greater dry matter digestibility of consumed pasturage among top grazers. Other experiments show that top-grazers herbage

was 10.9% greater in dry matter digestibility than bottom-grazed herbage (2). In a digestion trial where the top and bottom portions of alfalfa were fed separately to heifers, the TDN was much greater for top than for bottom growth (6).

There was not a shortage of pasture for bottom grazers; the ungrazed residue (mainly stems) averaged 711 lb. of dry matter per acre when these grazers were rotated to another pasture. Although the bottom grazers were never forced to graze all the available herbage, the intake of dry matter by the bottom grazers, as determined by the chromic oxide technique, was 15% lower than for top grazers.

The greater effect of supplements in stimulating milk production among bottom grazers was due to a deficiency of energy among bottom grazers. The bottom grazers had limited opportunity for selective grazing; therefore, the low digestibility of the ingested dry matter from the forage restricted milk production.

The results suggest that high acre and animal output may be attained by embodying the top- and bottom-grazing technique. High-producing animals may graze selectively in a fresh pasture until about one-half to two-thirds of the grazeable herbage is eaten. This encourages high animal output with limited grain feeding. Low-producing animals or dry stock may be used to graze the residue left by high producers. This scheme makes it possible to utilize herbage efficiently and the end result is a high return per acre. Many practical farmers in Europe are now using such a grazing plan.

The stocking rates–selective grazing interrelationships also suggest the need for more concentrate feeding as selective grazing decreases. Concentrate supplements also make it possible to utilize the herbage efficiently (limited selective grazing) without sacrificing output per animal.

Top and bottom grazing, as described in this paper, may be quite similar to light and heavy stocking rates, respectively. Stocking rates based on herbage availability and degree of selective grazing influence milk or meat production per animal. A relatively high stocking rate decreases production per animal but increases output per acre. Conversely, a relatively low stocking rate increases output per animal but reduces output per acre. The compromise between output per animal and output per acre has been reported by others (1, 2, 5, 9, 12, 14). Grazing schemes, such as forward-creep grazing, have stimulated growth of lambs (19), because this involves very light stocking (high selective grazing). The high liveweight gains of heifers grazing in fresh pastures ahead of milk cows (12) also embodies the principle of selective grazing.

REFERENCES

(1) Blaser, R. E., Bryant, H. T., Ward, C. Y., Hammes, R. C., Jr., Carter, R. C., and MacLeod, N. H. Symposium on Forage Evaluation: VII. Animal Performance and Yields with Methods of Utilizing Pasturage. Agron. J., 51: 238. 1959.

(2) Blazer, R. E., Hammes, R. C., Jr., Bryant, H. T., Hardison, W. A., Wontenot, J. P., and Engel, R. W. The Effect of Selective Grazing on Animal Output. Proc. 8th Intern. Grassland Congr. 601. 1960.

(3) BLASER, R. E., HAMMES, R. C., JR., BRYANT, H. T., KINCAID, C. M., SKRDLA, W. H., TAYLOR, T. H., AND GRIFFITH, W. L. The Value of Forage Species and Mixtures for Fattening Steers. Agron. J., 48: 508. 1956.

(4) GEUS, J. DE, AND HART, M. L. The Production and Behavior of Pasture Plants Under Ration Grazing. 6th Intern. Grassland Congr. Proc., 1: 371. 1952.

(5) HANCOCK, JOHN. The Conversion of Pasture to Milk. The Effect of Stocking Rate and Concentrate Feeding. J. Agr. Sci., 50: 284. 1958.

(6) HARDISON, W. A., LINKOUS, W. N., WARD, C. Y., BLASER, R. E., ENGEL, R. W., AND GRAF, G. C. The Intake and Digestibility of Nutrients in the Top and Bottom Portions of the Alfalfa Plants. J. Animal Sci., 14: 1238. 1955.

(7) HARDISON, W. A., AND REID, J. T. Use of Indicators in the Measurement of the Dry Matter Intake of Grazing Animals. J. Nutrition, 51: 35. 1953.

(8) HARDISON, W. A., REID, J. T., MARTIN, C. M., AND WOOLFOLK, P. G. Degree of Herbage Selection by Grazing Cattle. J. Dairy Sci., 37: 89. 1954.

(9) KINCAID, C. M., HUNT, R. E., AND LITTON, G. W. Age, Winter Gain, and Grazing Methods as Factors in Fat Steer Production on Pasture. Virginia Agr. Expt. Sta., Bull. 135. 1958.

(10) LAMBOURNE, L. J. A Comparison Between Rotational Grazing and Set-Stocking for Fat-Lamb Production. New Zealand J. Sci. Technol., 37: 555. 1956.

(11) LINE, C. Maximum Milk Production from Pasture. Proc. 8th Intern. Grassland Congr. 598. 1960.

(12) MCMEEKAN, C. P. Grazing Management and Animal Production. Proc. 7th Intern. Grassland Congr. 146. 1956.

(13) MEYER, J. H., LOFGREEN, G. P., AND HULL, J. L. Selective Grazing by Sheep and Cattle. J. Animal Sci., 16: 766. 1957.

(14) MOTT, G. O. Grazing Pressure and the Measurement of Pasture Production. Proc. 8th Intern. Grassland Congr. 606. 1960.

(15) PIEPER, REX, COOK, C. WAYNE, AND HARRIS, LORIN E. Effect of Intensity of Grazing upon Nutritive Content of the Diet. J. Animal Sci., 18: 1031. 1959.

(16) RAYMOND, W. F., MINSON, D. J., AND HARRIS, C. E. The Effect of Management on Herbage Consumption and Selective Grazing. Proc. 7th Intern. Grassland Congr. 123. 1956.

(17) REID, J. T., WOOLFOLK, P. G., HARDISON, W. A., MARTIN, C. M., BRUNDAGE, A. L., AND KAUFMAN, R. W. A Procedure for Measuring the Digestibility of Pasture Forage Under Grazing Conditions. J. Nutrition, 46: 255. 1952.

(18) SJOLLEMA, B. The Most Favorable Method of Grazing in Connection with the Requirements of Dairy Cows. Proc. 5th Intern. Grassland Congr. 191. 1949.

(19) WHITEHOUSE, A. W. It's Rotational Forward Creep Grazing. Agriculture, 66(8): 354. 1959.

COMPARISON OF CONTINUOUS AND ROTATIONAL GRAZING OF THREE FORAGE MIXTURES BY DAIRY COWS[1]

H. T. BRYANT, R. E. BLASER, R. C. HAMMES, JR., AND W. A. HARDISON
Departments of Agronomy and Dairy Science
Virginia Agricultural Experiment Station, Blacksburg

SUMMARY

Continuous and rotational grazing of three mixtures, alfalfa-orchardgrass, Ladino clover–orchardgrass, and Kentucky bluegrass–white clover–bird's-foot trefoil, were compared as pasturage for dairy cows during two grazing seasons.

The average daily FCM production by cows continuously and rotationally grazing alfalfa-orchardgrass was 27.2 and 28.4, Ladino clover–orchardgrass 27.4 and 23.3, and bluegrass–white clover–bird's-foot trefoil 25.4 and 25.6 lb., respectively, during the 21 wk. alfalfa-orchardgrass was grazed each year. Mean weekly persistency of FCM during the above period was 98.4, 99.0, and 97.2% for continuous grazing of alfalfa-orchardgrass, Ladino clover–orchardgrass, and Kentucky bluegrass–white clover, respectively, and 99.0, 97.4, and 98.9%, respectively, for the same mixtures grazed rotationally.

The milk production per acre was 6,204, 4,012, and 4,818 lb., respectively, from rotationally grazed alfalfa-orchardgrass, Ladino clover–orchardgrass, and bluegrass–white clover–bird's-foot trefoil, as compared to 4,802, 3,974, and 3,980, respectively, for the same mixtures grazed continuously.

The daily carrying capacity per acre was 30.0, 18.9, and 21.6% greater for the rotationally grazed alfalfa-orchardgrass, Ladino clover–orchardgrass, and bluegrass–white clover–bird's-foot trefoil pastures, respectively, than the continuously grazed pastures.

There was no significant difference in live weight gains between the cows for either the different grazing managements or forage mixtures.

Investigators are not in agreement on acre and animal output as affected by continuous and rotational grazing. Harrison *et al.* (8) found higher milk production per cow on continuous than on rotational grazing, while other investigators have shown higher production from rotational grazing (6, 10) or no differences (4, 5). Published results show discrepancies on acre returns; however, for species that are easily defoliated, milk production is higher with rotational than with continuous grazing (4, 9).

Objective of this study was to compare milk production under rotational and continuous grazing (on a per-animal and per-acre basis) for three mixtures, alfalfa-orchardgrass, Ladino clover–orchardgrass, and Kentucky bluegrass–Virginia white clover–bird's-foot trefoil.

EXPERIMENTAL PROCEDURE

The pastures consisted of 7.2 acres of alfalfa-orchardgrass, and nine acres each of Ladino clover–orchardgrass and Kentucky bluegrass–Virginia white clover–bird's-foot trefoil. Half of the area in each mixture was grazed continuously during both years; the other half was divided into ten equal strips with electric fences, and grazed rotationally. The rotationally grazed pastures

Received for publication May 11, 1961.

[1] This work was partially supported by the Old Dominion Foundation.

were managed as follows: the alfalfa-orchardgrass pastures were grazed when the alfalfa was at bud to 1/10th bloom, and grazed to approximately 3 in. or until most of the leafy growth was consumed; the Ladino clover–orchardgrass pasture was grazed from a height of 6 to 12 in. back to approximately 2 in. from the soil, and the bluegrass–white clover–bird's-foot trefoil pasture was grazed from a height of 4 to 7 in. back to approximately ¾ in. from the soil. An adequate supply of pasturage was maintained under both grazing managements. The desired stage of growth for both grazing managements was maintained by adjusting the stocking rate with additional grazers (2).

Each year there was a 28-day pregrazing standardization period. During the standardization period, cows were fed alfalfa-orchardgrass silage ad libitum and 1 lb. of a concentrate (16% total crude protein) to each 5 lb. of 4% fat-corrected milk (FCM). Immediately following standardization, groups of six cows were selected for similarity in milk production, stage of lactation, size and age, and assigned to the six treatments listed in Table 1. There were four and three cows, respectively, in 1958 and 1959, for each of the six pasture treatments, a total of 42 lactating Holstein cows. Concentrates and feed supplements were not fed during the grazing periods.

Milk weights were recorded twice daily and one-day composite samples were obtained once each month for analysis of milk fat according to the Babcock procedure. Milk production is reported as 4% fat-corrected milk (FCM). An index to the milk-stimulating effect of the pastures was determined by computing a persistency rating, based on the ratio between each week's and the prior week's production of the cows on a given pasture. Cows were weighed monthly in 1958, weekly in 1959, and for two consecutive days at the beginning and end of each grazing period.

There were four to six rotational grazing cycles each year and data on herbage yields are reported for the two rotational grazing cycles in 1959, when chromogen and chromic oxide determinations were made. Five random samples were taken from each of the rotationally grazed sublots immediately before and after grazing, and 15 samples were taken once a month from each of the continuously grazed pastures. Yield samples were obtained by mowing 1.5-ft. by 10-ft. strips with the mower blade set at ¾ in. above the soil surface. Estimates of herbage digestibility and dry matter intake were obtained from one cow per treatment in 1959 by the chromogen (13) and chromic oxide (7) techniques. Ten grams of chromic oxide was administered and fecal grab samples were collected after each milking. Fecal and forage samples were saved for chemical analyses.

The animal production data analyzed each year were obtained during the first 21 wk. of each grazing season; the period alfalfa-orchardgrass was grazed. Carrying capacity and milk production per acre were obtained each year for the entire grazing season.

EXPERIMENTAL RESULTS

Average daily milk production was 26.7 and 25.8 lb. for the continuous and rotational grazers, respectively, for the two 21-wk. grazing periods. Although

the differences were not significant, the average daily milk production was 6.9% greater for continuous than for rotational grazers in 1958; but there was no difference in 1959 between the two grazing methods. The over-all milk production averages indicate little difference between grazing methods; however, during both years the continuous grazer's milk production was greater on Ladino clover–orchardgrass after 6 wk., and less on alfalfa-orchardgrass after 11 wk. than the rotational grazers on these pastures. The average milk production of the cows for the grazing managements and pasture mixtures are shown (Figure 1).

Mean weekly milk production persistencies for the continuous grazers were 99.2 and 97.2%, as compared to 99.5 and 97.3% for the rotational grazers during the first 21 wk. in 1958 and 1959, respectively. Persistency of the continuous grazers on Ladino clover–orchardgrass was slightly higher than that of rotational grazers. However, persistency on the other pasture mixtures was slightly higher for rotational than for continuous grazers.

In 1958, the average milk production during the period all the pastures were grazed was 100, 87.4, and 93.2% of the production the last week of the pregrazing standardization period for the alfalfa-orchardgrass, Ladino clover–orchardgrass, and bluegrass–white clover–bird's-foot trefoil pastures, respectively. This was a statistically significant difference in maintenance of milk production between the cows grazing alfalfa-orchardgrass and those grazing Ladino clover–orchardgrass. Only small differences between pasture mixtures in maintenance of milk production were noted in 1959.

Average monthly milk yields and daily carrying capacity per acre for the two grazing seasons are given in Table 1. The average milk production per acre was 6,204, 4,012, and 4,818 lb. for rotational, as compared to 4,802, 3,974, and 3,980 for the continuous grazers on alfalfa-orchardgrass, Ladino clover–orchardgrass, and bluegrass–white clover–bird's-foot trefoil, respectively. The average daily carrying capacity per acre was 30.0, 18.9, and 21.6% greater, respectively, on the rotationally grazed alfalfa-orchardgrass, Ladino clover–orchardgrass, and bluegrass–white clover–bird's-foot trefoil pastures, than the continuously grazed pastures.

Digestibility and dry matter intake of the pasturage declined between the May-June and August-September grazing cycles (Table 2). Estimated dry matter digestibilities were 67.2 and 54.3%, respectively, for the continuously grazed pasture during the May-June and August-September grazing cycles, as compared to 68.3 and 56.1%, respectively, for the rotationally grazed pasturage. During the May-June grazing cycle, 3.79 and 4.46 lb. of dry matter was ingested by the continuous and rotational grazers, respectively, as compared to 3.45 and 3.19 lb., respectively, during the August-September grazing cycle.

There was an average of 681 and 132 lb., respectively, of dry herbage per acre for the May-June and August-September grazing cycles on the continuously grazed pastures. Dry matter yield per acre averaged 1,442 and 423 before and 481 and 132 lb. after grazing, respectively, for all rotationally grazed pastures during the May-June and August-September grazing cycles. The rotational

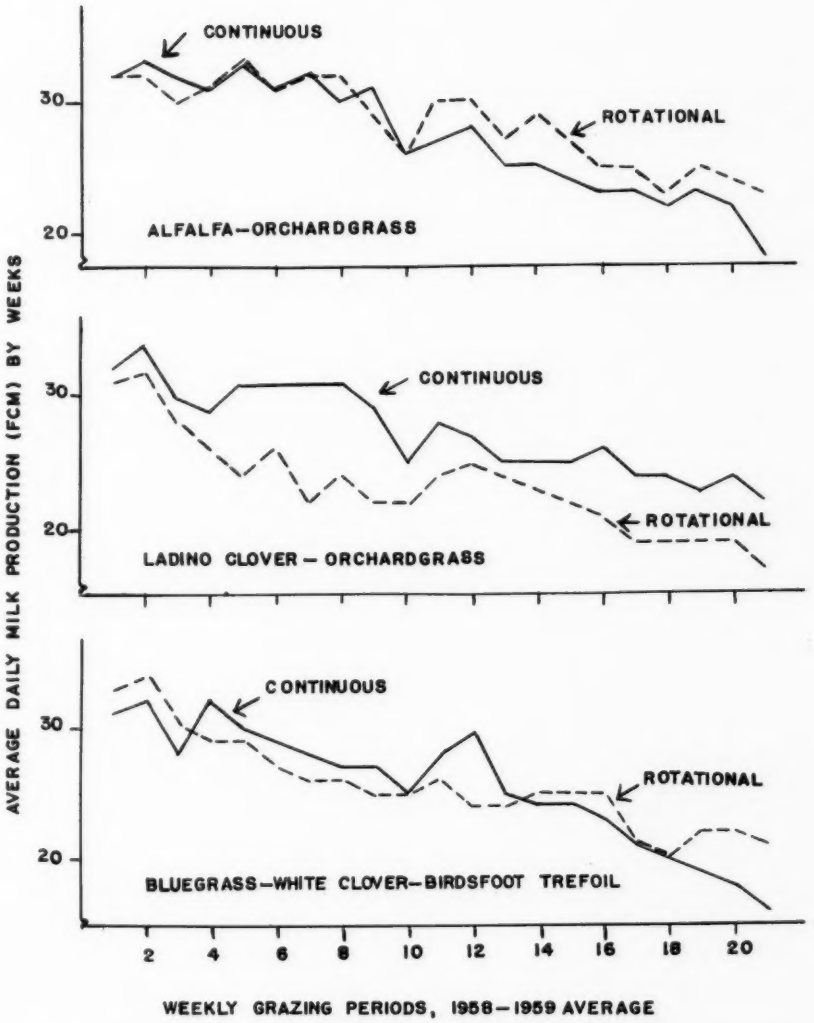


FIG. 1. Average daily production of 4% fat-corrected milk by weeks of cows continuously and rotationally grazing three pasture mixtures. Milk production in 1958 was significant at 5% level for forage.

TABLE 1

Cow-days' carrying capacity (1,000-lb. cow unit) and milk production per acre for the different grazing managements and pasture mixtures

Mixtures and grazing management	Months													
	April		May		June		July		August		Sept.		Oct.	Mean
	1958	1959	1958	1959	1958	1959	1958	1959	1958	1959	1958	1959[a]	1958	1958-1959
	Pounds 4% FCM per acre by months													
Alfalfa-orchardgrass														
Continuous	533	139	1,075	1,586	1,025	997	991	601	908	427	375	948	.....	800
Rotational	644	382	1,848	1,870	1,052	1,182	1,107	958	1,250	1,098	510	507	.....	1,034
Ladino clover–orchardgrass														
Continuous	447	106	1,011	915	806	794	874	467	897	344	780	338	168	603
Rotational	320	212	1,358	1,139	502	718	660	602	677	408	850	440	139	617
Kentucky bluegrass–white clover														
Continuous	407	109	942	1,075	757	775	838	674	778	357	722	200	325	612
Rotational	278	232	1,484	1,543	960	737	674	678	935	500	810	434	371	741
	Daily carrying capacity per acre													
Alfalfa-orchardgrass														
Continuous	1.96	0.96	1.16	1.50	1.22	0.98	1.18	0.77	1.20	0.65	1.22	1.85	.....	1.22
Rotational	2.17	2.84	2.07	1.78	1.23	1.18	1.24	1.08	1.62	1.40	1.37	1.05	.....	1.59
Ladino clover–orchardgrass														
Continuous	1.57	0.74	1.25	0.82	1.05	0.76	1.04	0.61	1.06	0.50	1.07	0.74	1.15	0.95
Rotational	1.16	1.54	1.93	1.17	0.92	0.82	0.93	0.79	0.95	0.74	1.27	1.42	1.04	1.13
Kentucky bluegrass–white clover														
Continuous	1.40	0.85	1.07	1.07	1.03	0.85	1.02	0.82	1.00	0.60	1.06	0.80	1.10	0.97
Rotational	0.94	1.58	1.71	1.57	1.51	0.80	0.95	0.81	1.30	0.72	1.25	0.93	1.32	1.18

[a] Pastures were plowed for another experiment in September, 1959.

TABLE 2

Estimated dry matter digestibilities and intakes for the different grazing managements and pasture mixtures

Pasture mixtures	Grazing management			
	Continuous		Rotational	
	May-June	Aug.-Sept.	May-June	Aug.-Sept.
	Estimated dry matter digestibility of ingested herbage			
Alfalfa-orchardgrass	67.8	55.0	69.0	56.9
Ladino clover–orchardgrass	67.9	53.7	69.2	54.0
Kentucky bluegrass–white clover	65.9	54.1	66.7	57.4
Mean	67.2	54.3	68.3	56.1
	Estimated daily dry matter intake (per 100-lb. live weight)			
Alfalfa-orchardgrass	3.40	3.42	4.96	3.26
Ladino clover–orchardgrass	4.12	2.96	4.09	3.24
Kentucky bluegrass–white clover	3.84	3.96	4.32	3.08
Mean	3.79	3.45	4.46	3.19

grazers consumed 67 and 69%, respectively, of the herbage the first and second grazing cycles.

The protein content of the herbage averaged 18.2 and 16.8%, respectively, when cows were moved into a pasture and 13.06 and 14.73% when rotated out of a pasture for the May-June and August-September grazing cycles (Table 3). The average protein content was 14.6 and 14.0%, respectively, for the herbage in the continuously grazed pastures for the two grazing cycles. The continuous grazers' pasturage during both grazing cycles was slightly higher in crude fiber and nitrogen-free extract, and lower in ash, than the rotated pastures before grazing. For both grazing managements the average crude protein, ether extract, ash and crude fiber was higher and the nitrogen-free extract lower for the May-June than the August-September grazing cycle.

Alfalfa and Ladino clover were 48 and 19%, respectively, of the stand on the rotationally grazed pastures the last year, as compared to 17 and 13%, respectively, for the continuously grazed pastures. Bird's-foot trefoil averaged 12% of the stand on the rotationally grazed bluegrass–white clover–bird's-foot trefoil pastures, and less than ½% on the continuously grazed pastures. There was 50% more white clover on the continuously than on the rotationally grazed bluegrass–white clover–bird's-foot trefoil pastures. Weeds averaged 30% of the alfalfa-orchardgrass stands under continuous, as compared to less than 9% under rotational grazing. Bluegrass pastures, however, contained few weeds under both grazing managements.

DISCUSSION

The results of this study show no significant difference in milk production per cow under continuous and rotational grazing. The milk production or output per cow is usually associated with the amount and digestibility of the ingested dry matter. The amount of dry matter ingested is influenced by stocking rate, in that a light stocking rate augments animal output by increasing herbage availability and dry matter intake, as compared with a heavier stocking

TABLE 3

Average composition of herbage for the different grazing managements and pasture mixtures

	Herbage composition (%)									
	Crude protein		Ether extract		Crude fiber		Ash		Nitrogen-free extract	
	May-June	Aug.-Sept.	May-June	Aug.-Sept.	May-June	Aug.-Sept.	May-June	Aug.-Sept.	May-June	Aug.-Sept.
Continuous grazing										
Alfalfa-orchardgrass	13.61	14.45	3.48	2.99	31.57	30.53	8.92	7.35	42.42	44.68
Ladino clover–orchardgrass	14.50	12.73	3.61	3.82	30.60	27.18	8.16	7.86	43.12	48.40
Kentucky bluegrass–white clover	15.63	14.87	4.09	3.47	28.72	26.22	8.43	8.05	43.13	47.39
Rotational grazing										
Alfalfa-orchardgrass										
Before grazing	21.72	18.18	3.82	2.53	24.85	25.12	9.26	8.34	40.34	45.83
After grazing	14.47	13.94	2.49	2.31	32.54	39.42	7.64	7.90	42.86	45.64
Ladino clover–orchardgrass										
Before grazing	16.75	16.75	4.30	3.66	27.35	24.31	9.12	8.18	42.48	47.10
After grazing	12.69	14.88	3.54	3.31	31.71	25.40	8.81	14.00	43.25	42.41
Kentucky bluegrass–white clover										
Before grazing	16.19	15.38	3.91	3.78	27.75	26.72	8.93	7.62	43.23	46.50
After grazing	12.02	15.38	3.47	2.91	30.64	28.29	7.93	8.02	45.94	45.40

rate or grazing pressure (1, 11, 12). Index data on dry matter ingested show little difference in intake between the cows for either the two grazing managements or three grazing mixtures. Furthermore, there was little difference in the digestibility of the herbage dry matter ingested by the continuous and rotational grazers among the three mixtures. These small differences in dry matter intake and digestibility suggest that stocking rate in terms of available pasturage was reasonably well controlled for the two grazing managements and the three pasture mixtures. Also, the high weekly milk persistency per cow obtained from pasturage alone and the slight increase in live weight of the cows indicate that adequate pasturage of good quality was ingested during both grazing seasons.

There was considerably lower milk production and carrying capacity per acre for continuous as compared with rotational grazing. The low production per acre with continuous grazing should not be entirely attributed to inefficient use of the pasturage, as with the put-and-take technique (2); the stocking rate is adjusted in terms of herbage availability. The higher production per acre with rotational grazing than with continuous grazing may be due to changes in botanical composition of the mixtures and the effect of less frequent grazing on yields of pasturage. There was a greater yield of grasses and legumes and less weeds from rotational as compared to continuously grazed pastures. The poor stands of Ladino clover, irrespective of grazing management, may partially explain the slight difference in yield per acre between the rotational and continuously grazed Ladino clover–orchardgrass pastures.

Yield of pasturage generally increases as the ratio of rest to grazing increases. With less frequent grazing, rotationally grazed plants may be able to store more organic food in the stubble and underground parts of the plant than the continuously grazed plants. The stored organic food reserves provide a source of energy for fast regrowth (14). Rate of growth is also dependent upon the amount of available light that is intercepted (3). The interception of incident light may, during certain periods, be a factor affecting forage growth. The utilization of light (photosynthesis) may be more consistent on continuously than rotationally grazed pastures. The competition for light between leaves is usually not a limiting growth factor on continuously grazed pastures. However, with rotational grazing, the plant's top growth may accumulate sufficiently between grazings to shade the lower leaves. Shaded leaves may utilize more organic food in respiration than they synthesize. The greater yield per acre in this experiment under rotational than continuous grazing indicates that the effect of shading on the lower leaves was not sufficient to offset the advantage of the larger leaf area that accumulated between grazing periods.

REFERENCES

(1) Blaser, R. E., Hammes, R. C. Jr., Bryant, H. T., Hardison, W. A., Fontenot, J. P., and Engel, R. W. The Effect of Selective Grazing on Animal Output. Proc. 8th Intern. Grassland Congr., 601. 1960.

(2) Blaser, R. E., Hammes, R. C. Jr., Bryant, H. T., Kincaid, C. M., Skrdla, W. H., Taylor, T. H., and Griffeth, W. L. The Value of Forage Species and Mixtures for Fattening Steers. Agron. J., 48: 508. 1956.

(3) Brougham, R. W. Effect of Intensity of Defoliation on Regrowth of Pasture. Australian J. Agr. Research, 7: 377. 1956.

(4) Brundage, A. L., and Petersen, W. E. A Comparison Between Daily Rotational Grazing and Continuous Grazing. J. Dairy Sci., 35: 623. 1952.

(5) Davis, R. R., and Pratt, A. D. Rotational vs. Continuous Grazing with Dairy Cows. Ohio Agr. Expt. Sta., Research Bull. 778. 1956.

(6) Hancock, John, and McMeekin, C. P. Studies of Grazing Behavior in Relation to Grassland Management. III. Rotational Compared with Continuous Grazing. J. Agr. Sci., 45: 96. 1954.

(7) Hardison, W. A., and Reid, J. T. Use of Indicators in the Measurements of Dry Matter Intake of Grazing Animals. J. Nutrition, 51: 35. 1953.

(8) Harrison, C. M., Williams, G., and Fischer, W. N. Continuous and Rotation Grazing of a Legume-Grass Mixture with Dairy Cows. J. Am. Soc. Agron., 40: 357. 1948.

(9) Hodgson, R. E., Grunder, M. S., Knott, J. C., and Ellington, E. V. A Comparison of Rotational and Continuous Grazing of Pastures in Western Washington. Washington Agr. Expt. Sta., Bull. 294. 1934.

(10) Lundquist, N. S., Law, A. A., Ashworth, O. S., and Wolberg, F. B. A Comparison of Rotational and Continuous Grazing of Pastures in Eastern Washington. Washington Agr. Expt. Sta., Bull. 470. 1945.

(11) Pieper, Rex, Cook, C. Wayne, and Harris, Lorin E. Effect of Intensity of Grazing upon Nutritive Contents of the Diet. J. Animal Sci., 18: 1031. 1959.

(12) Raymond, W. F., Minson, D. J., and Harris, C. E. The Effect of Management on Herbage Consumption and Selective Grazing. Proc. 7th Intern. Grassland Congr., 123. 1956.

(13) Reid, J. T., Woolfolk, P. G., Hardison, W. A., Martin, C. M., Brundage, A. L., and Kaufman, R. W. A Procedure for Measuring the Digestibility of Pasture Forage Under Grazing Conditions. J. Nutrition, 46: 255. 1952.

(14) Sprague, V. G., and Sullivan, J. T. Reserve Carbohydrates in Orchardgrass Clipped Periodically. Plant Physiol., 25: 92. 1950.

THYROID SUPPLEMENTATION AT PEAK OF LACTATION[1, 2]

H. A. TUCKER AND R. P. REECE
Department of Dairy Science
New Jersey Agricultural Experiment Station, New Brunswick

SUMMARY

The daily feeding of three additional pounds of grain plus 1.2 g. thyroprotein per 100 lb. of body weight for 13 days to ten dairy cows at the peak of lactation increased milk production 9.9%. The daily feeding of three additional pounds of grain without thyroprotein for 13 days to eight similar dairy cows increased milk production only 1.1%. An analysis of variance showed that the between-group comparisons were highly significant ($P < 0.01$). The results suggest that thyroid secretion rate may be a limiting factor in milk secretion at the peak of lactation.

It has been generally assumed that the main limiting factor in milk production at the peak of lactation is udder capacity. Tucker *et al.* (3) have indicated, however, that maximum udder holding capacity, in terms of ability to secrete against intramammary pressure and/or any inhibitory effect of milk constituents at the peak of lactation, is not attained until 35 hr. after milking. Two alternative possibilities exist to explain this situation: Either the cells of the mammary parenchyma receive a prolonged maximal amount of stimulation and they continue to secrete for an extended period of time at an ever-decreasing rate, or the cells do not receive a maximal amount of stimulation and it requires many hours to attain maximal udder capacity. It was thought that if cows were given additional stimulation, and if milk production increased, then it could be concluded that factors in addition to udder capacity limit milk production at the peak of lactation. From the practical standpoint, it would be desirable to stimulate the udder to reach its maximum holding capacity within normal milking intervals.

EXPERIMENTAL PROCEDURE

Twelve purebred Jersey and eight purebred Brown Swiss cows were used and conditions were maintained as nearly normal as possible. The animals were grouped, with the exception of four first-calf heifers, which were randomly assigned two to each group, according to the previous year's milk production. Two to four weeks usually elapsed between calving and the beginning of an experiment. No animal was started on experiment until the udder was free from edema, the cow was on full feed, and her milk production had levelled off, thus indicating that peak of lactation had been attained. Two cows were lost from Group I before the experiment was completed.

The grain ration for all cows was a high-energy feed (74% TDN), fed throughout the experiment at a 1:3 grain-to-milk ratio. Corn silage and

Received for publication March 17, 1961.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University, Department of Dairy Science, New Brunswick.

[2] This study was supported by a grant from the Frelinghuysen Foundation.

alfalfa hay were fed ad lib. Seven consecutive days at the peak of lactation constituted the control period.

The cows in Group I received daily three additional pounds of the high-energy-grain ration during the 14-day experimental period. On the first day of the experimental period the cows in Group II were fed three additional pounds of grain containing either 40 g. (for Jerseys) or 50 g. (for Brown Swiss) of thyroprotein (protamone[3]). This was done in an attempt to elevate rapidly the thyroxine pool (1). On the second day of the experimental period and during the following 12 days the cows in Group II received the extra allowance of grain plus 1.2 g. of thyroprotein per 100 lb. of body weight.

Milk production on the highest seven consecutive days during the treatment period was used to represent the maximum response to extra feed and extra feed plus thyroprotein. Within each cow the average control value was subtracted from the average treatment value. An analysis of variance was carried out on the means of the between-group values according to Snedecor (2).

RESULTS AND DISCUSSION

The average daily production during the control period was 41.6 lb. for cows in Group I and 48.1 lb. for cows in Group II. Four of eight cows in Group I increased in milk production; whereas, all cows in Group II increased in production. The cows were quite variable as to number of days required before maximum response occurred. Of four cows in Group I showing an increase in production, two responded maximally during the first seven days and two during the last seven days of the experimental period. Seven cows in Group II attained a maximal response before the experiment was half over and the remaining three cows reached their maximum during the second half of the experiment.

The average percentage differences between control and experimental periods were 1.1 (−4.7 to +6.5) and 9.9 (+3.4 to +18.4) for Group I and Group II cows, respectively. The mean differences between the control and experimental periods for each cow within group are given in Table 1. The between-group comparisons were highly significant ($P < 0.01$).

The results suggest that thyroid secretion rate may be a limiting factor in milk secretion at peak of lactation. The magnitude of response of thyroprotein-fed cows gives some indication of the degree to which thyroid secretion rate was a limiting factor at peak of lactation. Moreover, it is possible that factors other than udder capacity and thyroid secretion rate are limiting milk production at lactational peak. Such possibilities should be explored.

Increasing the level of circulating thyroidal hormone at peak production must have increased the rate of milk synthesis, since there is no evidence that thyroprotein feeding increases the number of secretory cells. The secretion of greater quantities of milk results in increased intramammary pressure, and it is suggested that increasing the amount of circulating thyroidal hormone

[3] The Protamone was generously supplied by Agric-Tech, Inc., Kansas City, Missouri, through the courtesy of Mr. Bruce Varney.

TABLE 1

Effect of extra feed and extra feed plus thyroprotein on milk production of cows at peak of lactation

Extra feed [a]				Extra feed plus thyroprotein [b]			
	Av. daily milk production				Av. daily milk production		
Cow	Control period (lb.)	Change in experimental period (lb.)	Change in experimental period (%)	Cow	Control period (lb.)	Change in experimental period (lb.)	Change in experimental period (%)
A	45.5	+2.3	+5.1	I	53.6	+ 2.4	+ 4.5
B	44.3	−0.8	−1.8	J	48.6	+ 3.4	+ 7.0
C	44.5	+0.6	+1.3	K	41.2	+ 7.6	+18.4
D	36.9	+1.5	+4.1	L	51.4	+ 7.7	+15.0
E	27.7	−1.3	−4.7	M	44.2	+ 3.2	+ 7.2
F	45.9	+3.0	+6.5	N	38.0	+ 1.3	+ 3.4
G	40.9	−0.4	−1.0	O	62.0	+ 6.4	+10.3
H	47.1	−0.4	−0.8	P	51.5	+ 3.1	+ 6.0
				Q	60.3	+10.1	+16.7
				R	30.2	+ 3.1	+10.3
Av.	41.6	+0.6	+1.1	Av.	48.1	+ 4.8**	+ 9.9

** Significant at the 0.01% level when compared to extra feed.
[a] Group I.
[b] Group II.

enables the epithelial cells of the mammary gland to secrete against a higher intramammary pressure.

ACKNOWLEDGMENT

The assistance of Mr. Clifton J. Yard is gratefully acknowledged.

REFERENCES

(1) PREMACHANDRA, B. N., AND TURNER, C. W. Elevated Thyroxine Pool and Lactation. J. Animal Sci., 18: 1564. 1959.

(2) SNEDECOR, G. W. Statistical Methods. 5th ed. Iowa State College Press, Ames. 1956.

(3) TUCKER, H. A., REECE, R. P., AND MATHER, R. E. Udder Capacity Estimates as Affected by Rate of Secretion and Intramammary Pressure. J. Dairy Sci., 43: 880. 1960.

METABOLISM OF RADIOSTRONTIUM IN YOUNG PIGS AND IN LACTATING RATS FED STABLE STRONTIUM [1]

JOHN C. BARTLEY [2] AND ELWOOD F. REBER
College of Veterinary Medicine
University of Illinois and Illinois Agricultural Experiment Station, Urbana

SUMMARY

Lactating rats fed stable strontium retained more radiostrontium, and carcasses of their young contained significantly more radiostrontium, than the lactating rats and their offspring which did not receive stable strontium. Results of these experiments were compared to the effect of stable strontium on the absorption of radiostrontium observed in other species. The effect of feeding stable strontium for ten days before the radiostrontium meal was not significantly different from feeding stable strontium simultaneously with radiostrontium.

Feeding Hampshire pigs 21 days of age a ration containing 0.67% stable strontium, with 0.16 or 0.89% calcium, increased the amount of radiostrontium in the feces fourfold during the seven days after administration of radiostrontium. The amount of the absorbed radiostrontium appearing in the urine of the pigs fed strontium was about twice that of pigs not receiving strontium. The concentration of calcium in the diet had no marked effect on the quantity of radiostrontium in the urine and feces. Feeding stable strontium reduced the skeletal retention of radiostrontium three- to fourfold.

There has been an intensive search for agents which will reduce the absorption and retention of radiostrontium, one of the more dangerous elements in atomic fallout. However, until recently there were few data available on the effect of stable strontium on the absorption and retention of radiostrontium. Hamilton (7) reported that stable strontium added to the diet of rats had no effect on the absorption of radiostrontium added simultaneously. This work has been confirmed in rats, dairy cows, and goats (4, 19, 24).

A knowledge of strontium metabolism in swine would be useful, since swine are an important source of food for man and have a digestive system more comparable to that of man than any other animal except possibly the primate. However, only two studies have employed swine as the experimental animal (1, 6).

Previous studies (3) have shown that young pigs fed adequate calcium tolerated 0.67% strontium in the diet for at least 5 wk. The absorption of radiostrontium was reduced 60 to 80% in pigs which had received a ration containing stable strontium for a few weeks before the addition of radiostrontium to the ration. The metabolism of radiostrontium in pigs fed rations containing 0.16 or 0.89% calcium, with 0.67% or without strontium, is reported in the present article.

Received for publication September 19, 1961.

[1] Study submitted as partial fulfillment of the requirements for the degree of Master of Science in Veterinary Medical Science.

[2] Present address: Department of Pharmacology, Physiology, and Biochemistry, School of Veterinary Medicine, University of California, Davis.

Differences in age and species of the experimental animals, and in the timing of administration of stable and radiostrontium, may account for the differences in absorption of radiostrontium by pigs as compared to other species. A second experiment was designed to test the effect of feeding stable strontium before and simultaneously with the radiostrontium meal. Lactating rats were used so that possible effects on the excretion of radiostrontium in milk could be detected.

MATERIALS AND METHODS

In the experiment using four litter-mate Hampshire pigs, the feeding procedure and rations were the same as those used previously (3). Each pig was fed one of the following experimental rations for 5 wk., starting at 3 wk. of age: (1) 0.89% calcium and no strontium, (2) 0.67% strontium and 0.16% calcium, (3) 0.89% calcium and 0.67% strontium, (4) 0.16% calcium and no strontium.

Each pig was given 5 ml. of a solution containing 225 μc. of strontium^{89} (strontium^{90} contamination less than 10%), mixed into a portion of the food at the start of the 4th wk. of the experiment. Food free of radiostrontium was added to the feed pans in small quantities until they registered no more than 100 counts per minute (c.p.m.) at the surface.

Urine and feces were collected every morning for 1 wk. after the feeding of radiostrontium. The pigs were killed at the end of the 5-wk. experimental period, 1 wk. after the radiostrontium was fed. The right femur was taken from each pig for determination of the radiostrontium content.

In the second experiment, nine female Holtzman rats were allotted at random to three treatment groups at parturition. The treatment groups were: (1) laboratory feed [3] throughout the experiment, (2) laboratory feed containing 0.5% stable strontium throughout the experiment, (3) laboratory feed until ten days postparturition and then laboratory feed containing 0.5% stable strontium. The laboratory feed was ground in a hammer mill. Strontium was added as the chloride salt. The calcium content of the laboratory feed was 1.3%. The rats were housed singly in wire mesh cages so that all excreta from each cage could be collected.

The young were taken from the adults for 24 hr. nine days after parturition. On Day 10 the adult rats were offered the feed containing 20 μc. of radiostrontium (35% strontium^{89} and 65% strontium^{90}). Ten grams of laboratory feed free of radiostrontium were placed in the feed jars. This procedure was repeated until no more than 100 c.p.m. could be detected in the jars. Wasted food containing radiostrontium was caught on paper under the cages and replaced in the food jars. The consumption of the total dose of radiostrontium took less than 4 hr. The young were placed with the dam after she had consumed all the feed which contained radiostrontium.

Young rats from each litter were killed on each of the five days following the feeding of radiostrontium to the dams. Usually two were killed each day, but variation in the number was necessary with the small and very large litters,

[3] Purina Lab. Chow. Ralston Purina Company, St. Louis 2, Missouri.

to provide about the same number of samples each day over the five-day period. The mother rats were killed the fifth day following the radiostrontium meal. Urine and feces from each cage were collected for the five-day period.

The rat carcasses, the pig femurs and excreta collected in both experiments were digested in concentrated nitric acid as described by Comar (5). After at least 21 days, the quantity of radiostrontium in each of the samples was determined by comparing the activity in 5-ml. aliquots of the test sample with that of 5-ml. aliquots of known percentages of the radiostrontium dose counted on the same day. All samples were counted at the same geometry in Petri dishes 5 cm. in diameter. The counting rate was determined from the time required to accumulate 10,000 preset counts on a decade scaler.[4]

RESULTS AND DISCUSSION

The results of the studies with pigs, summarized in Table 1, indicate the action of stable strontium on intestinal absorption. The most striking result

TABLE 1

Percentage of the oral dose of radiostrontium recovered in feces, urine, and femurs of pigs

	Ration			
	0.16% Ca	0.89% Ca	0.16% Ca+ 0.67% Sr	0.89% Ca+ 0.67% Sr
Urinary excretion	8.3	6.0	3.4	3.5
Fecal excretion	15.4	20.0	80.7	76.8
Retained in one femur	3.2	3.4	0.5	1.3
Total bone retention [a]	75.2	64.3	9.9	25.3
Total retained [b]	76.3	74.0	20.0	19.9

[a] Calculated from the percentage of the dose per gram of femur on the basis that the total bone of the skeleton is 10% of the body weight (15).

[b] Calculated by subtracting the urinary excretion from the total dose absorbed.

was the increased amount of radiostrontium in the feces of those pigs fed stable strontium. The magnitude of the differences in the amount of radiostrontium in the feces is best seen in Figure 1, in which the daily fecal excretions are plotted. Feeding 0.67% stable strontium, with 0.16 or 0.89% calcium, increased the amount of radiostrontium in the feces fourfold during the seven days after the administration of radiostrontium. A reduction in the amount of radiostrontium absorbed by the pigs fed strontium resulted in a lower percentage of the radiostrontium intake being eliminated in the urine or retained in bone. When the amount of radiostrontium excreted in the urine is expressed as a percentage of radiostrontium absorbed, the urinary excretion of the absorbed radiostrontium by pigs fed the rations containing 0.67% stable strontium was almost twice that of the pigs fed rations devoid of stable strontium (Table 1). The percentage of radiostrontium absorbed was calculated by subtracting the total in the feces from 100%. It was assumed that negligible amounts of the

[4] Nuclear-Chicago Scaler, Model 181A. Nuclear-Chicago Corporation, Chicago, Illinois.

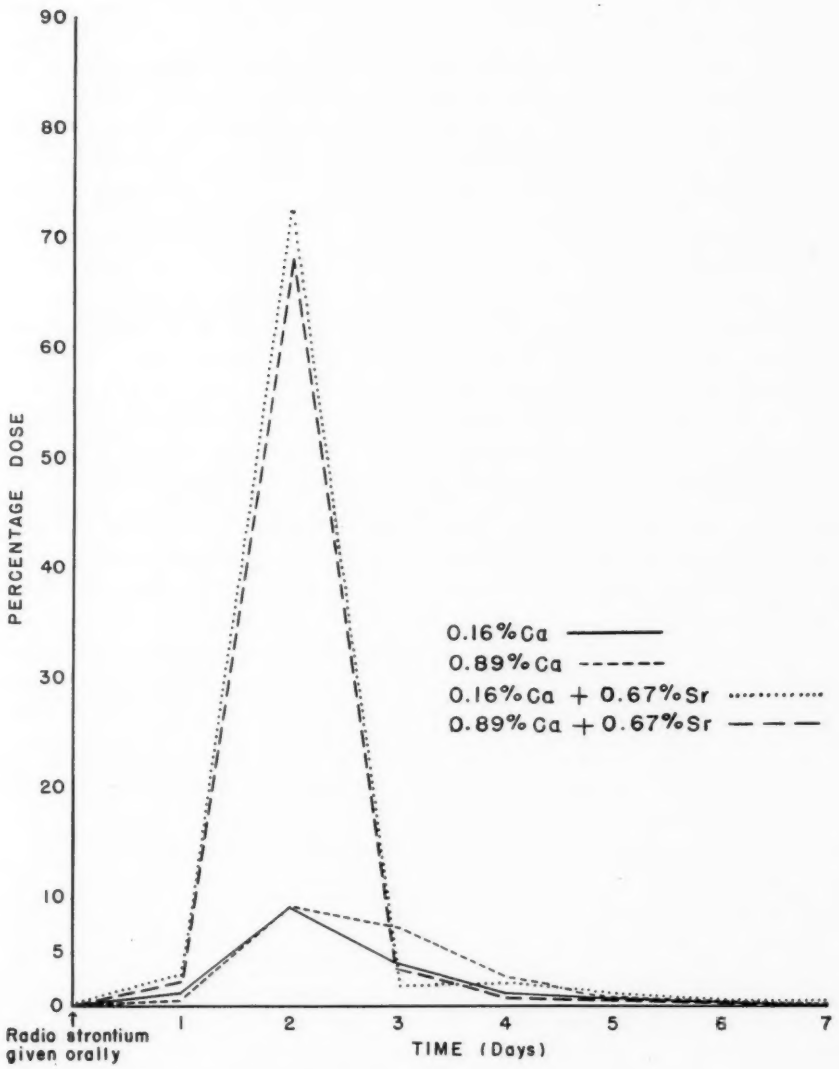


FIG. 1. Daily fecal excretion of radiostrontium.

radiostrontium which had been absorbed were excreted into the intestine (21).

The percentage of the dose remaining in a femur seven days after administration of radiostrontium reflected the quantity of radioactive material absorbed and the state of bone metabolism in the animal. Feeding stable strontium and 0.89% calcium reduced the femoral retention of radiostrontium by one-half. The bone abnormalities which were a result of feeding 0.16% calcium appear to be responsible for the slightly reduced femoral content of radiostrontium.

The retention of radiostrontium in the whole skeleton was calculated from the quantity of radiostrontium per gram of bone, assuming that about 10% of the body weight was bone (15). It was also estimated by subtracting the amount of radiostrontium that appeared in the urine from the total absorbed. The latter method includes the radiostrontium not incorporated into bone. Both values of radiostrontium retention are given in Table 1. The discrepancies between the values are probably due to variation between pigs in the percentage of bone weight to body weight. The pigs fed strontium retained the least radiostrontium by either calculation. The presence of stable strontium in the diet reduced the skeletal burden of radiostrontium three- to fourfold.

The percentage of the dose retained in bone after seven days was calculated from the dose absorbed and the bone retention. The pigs retained 83 to 93% of the radiostrontium absorbed when the retention was based on the urinary and fecal excretion. The pig fed 0.67% strontium and 0.16% calcium had the lowest retention, probably due to the marked changes in bone induced by feeding this ration. The other values for radiostrontium retention in bone are comparable to those reported in the literature when radiostrontium was given parenterally (18, 22).

There were slight differences in the rations used in the experiment with pigs other than the concentrations of strontium and calcium. The ration which contained 0.16% calcium had 1% more lactose, that which contained 0.67% strontium and 0.89% calcium provided 0.5% more carbonate, and that containing 0.89% calcium provided 0.4% more lactate than the other ration. Although carbonate, lactose, and lactate all have been shown to have an influence on strontium absorption (7, 12, 14, 16, 17, 23), it is not likely that the slightly higher concentrations provided were high enough to have an effect.

All the results agree with the concept that stable strontium in the diet of pigs markedly reduced the intestinal absorption and increased renal excretion of ingested radiostrontium. The amount of radiostrontium in one femur has been used frequently to compare the effectiveness of agents to reduce the absorption and retention of radiostrontium. The diets containing 0.67% stable strontium reduced the percentage of radiostrontium retained in the femur by 86% in the pig fed 0.16% calcium and by 61% in that fed 0.89% calcium. This reduction was greater than that observed with any of the many compounds tested by MacDonald *et al.* (13) or Palmer *et al.* (16, 17). Kawin (8) reported that stable strontium given intraperitoneally with strontium^{89} reduced the femoral retention of strontium^{89} by 30% in rats. Other workers found little reduction of the absorption of oral radiostrontium when stable strontium was

given simultaneously (7-9). These workers used levels of stable strontium much lower than those used here. The difference between the response of pigs and the response of rats and cows could be due to the higher concentration of strontium fed, species differences, or the feeding of strontium for 4 wk. before the radiostrontium meal.

Feeding stable strontium to female rats before or simultaneously with the radiostrontium meal produced no significant difference between the average percentage of radiostrontium in the carcasses of individual young. Including stable stronium in the ration resulted in a significant ($p = 0.01$) increase in the average percentage of radiostrontium retained by young rats, as compared to the retention of the radioisotope by young rats of the females fed the laboratory stock ration (Table 2).

TABLE 2

Average percentage of the maternal dose of radiostrontium in the filial carcasses

	Days after radiostrontium was fed				
	1	2	3	4	5
Rats fed commercial laboratory animal feed:					
Average	0.23 (6)[a]	0.21 (6)	0.28 (6)	0.25 (6)	0.26 (4)
Range	6.16–0.32	0.13–0.28	0.22–0.38	0.19–0.32	0.22–0.30
Rats fed commercial laboratory animal feed containing 0.5% strontium throughout experiment:					
Average	0.50 (6)	0.54 (6)	0.49 (7)	0.43 (5)	0.49 (2)
Range	0.42–0.60	0.25–0.78	0.40–0.71	0.30–0.60	0.35–0.62
Rats fed commercial laboratory animal feed containing 0.5% strontium simultaneously with radiostrontium meal and thereafter:					
Average	0.33 (5)	0.51 (5)	0.47 (6)	0.54 (5)	0.50 (3)
Range	0.26–0.40	0.34–0.65	0.40–0.60	0.38–0.74	0.35–0.60
Grand average	0.35	0.41	0.42	0.40	0.39

[a] Numbers in parentheses indicate the number of young in that sample.

The radiostrontium retained by the young rats increased from an average of 0.35% on Day 1 to 0.41% on Day 2, and stayed at this level for the remainder of the five-day period. An apparent trend toward a gradual increase during the test period was not statistically significant. Since the level of radiostrontium in the young was dependent upon the level of radiostrontium in the milk, the latter level must have been rather constant during the five days after the radiostrontium meal. Due to the practice of coprophagy (2), substances in the diet of mature rats may be subjected to digestive and absorptive processes more than once. The ingestion of feces by the dams may have maintained the intake of radiostrontium for the entire experimental period.

Since there was little day-to-day variation among litters of the same treatment, the total radiostrontium retained by each litter gives an indication of the percentage of radiostrontium secreted in the milk of the dam. Data on the percentage of radiostrontium recovered from the carcasses of the young, and on the percentage of radiostrontium retained by the dam five days after the radiostrontium meal, are summarized in Table 3 for each dietary group.

TABLE 3

Percentage of radiostrontium recovered from the carcasses of female rats and their offspring on the fifth day following a radiostrontium meal

	Treatment [a]		
	No strontium	Strontium [b]	Strontium [c]
Retained by the young	2.27	4.28	3.74
Retained by the female	0.85	5.24	1.46

[a] All rats were fed a commercial laboratory ration.
[b] Dams fed the laboratory ration containing 0.5% strontium throughout the experiment.
[c] Dams fed the laboratory ration containing 0.5% strontium starting at the time the radiostrontium was fed.

The percentage of radiostrontium found in the excreta was unrealistic in some cases (over 100%), but it can be calculated by subtracting the sum of the percentage of radiostrontium recovered from the young and the dam from 100% (Table 3). Unrealistic values for fecal excretion have been reported by other workers (23). The much greater radioactivity of the fecal material required high dilutions before counting, which would multiply the inherent error.

Feeding stable strontium caused an increase in the quantity of radiostrontium retained in the body and secreted in milk of the dam. The effect appeared to be the same when the stable strontium was fed before, or at the same time as, the radiostrontium meal. Increased absorption of radiostrontium in the presence of stable strontium could explain both of these results.

Increased absorption of radiostrontium in the presence of stable strontium is inexplicable at present and contrary to the response shown by young pigs. Rubanovskaya and Ushakova (19) reported no consistent effect of stable strontium on the retention of radiostrontium by rats. The absorption and retention of radiostrontium via the intestine of rats is certainly not comparable to those of animals not practicing coprophagy (2). Wasserman *et al.* (24) and Bell *et al.* (4) found no significant effect of stable strontium on the excretion of radiostrontium in cows' and goats' milk.

Results of the experiment on pigs indicate that at least a portion of calcium and strontium must be absorbed from the intestine by different mechanisms. Dietary calcium did not have the same effect as an equal amount of strontium in the diet. Kornberg (10) proposed that elements in the same periodic group as calcium would reduce absorption of radiostrontium to a similar degree. If this were true, the pig fed 0.67% strontium and 0.89% calcium should have absorbed the least amount of radiostrontium. Actually, this pig absorbed more radiostrontium than the pig fed 0.67% strontium and 0.16% calcium. According to Schachter and Rosen (20) and Kawin (8), strontium had no effect on the transport of calcium across the intestinal barrier. This relationship appears to hold for the action of calcium on strontium absorption (8). Kornberg (9) suggested that dilution of radiostrontium by stable strontium would be effective only if a specific acceptor substance was saturated with stable strontium before giving the radiostrontium. A reaction of this sort may have been important in the immature pigs. The reduced absorption of radiostrontium when stable

strontium was in the diet could be due to dilution of the radiostrontium by the stable strontium during the absorptive process or a specific action by stable strontium on the absorptive mechanism during the prefeeding period. It would appear that neither of these mechanisms was important in the mature rats.

Different mechanisms of absorption of radiostrontium, due to age or species differences or both, might explain the differences demonstrated between immature pigs and mature rats. Lengemann *et al.* (11) reported age differences in the degrees of retention of radiostrontium by cattle and human beings fed milk-containing and milk-free diets. The strontium-calcium ratio was lower in the rations for the rats than those for the pigs. This difference in diet may have contributed to the disparity between rats and pigs in the degree of retention of radiostrontium.

If the disparity is due to species differences, the implications are very interesting. The digestive system of man is anatomically and physiologically more like that of swine than of other animals except the subhuman primates. If dietary additives influence absorption of radiostrontium in the pig in a manner different from that of other animal species, might not this difference be true of man also?

ACKNOWLEDGMENT

The authors express their appreciation to Dr. H. W. Norton, Department of Animal Science, for aid in statistical analyses, and to Dr. L. Michaud, Veterinary Research Department, Merck and Company, Inc., Rahway, New Jersey, for supplying the B-complex vitamins for this study.

REFERENCES

(1) Anonymous. Semiannual progress report for July 1, 1955, to December 31, 1955. Fission Product Metabolism in Animals. U. S. AEC Doc. ORO-150: 20. 1955.

(2) Barnes, R. H., Fiala, G., Delaney, K., and Caplan, E. Coprophagy, Refection, and the Influence of Antibiotics in the Rat. Federation Proc., 18: 516. 1959.

(3) Bartley, J. C. Stulies of Metabolism and Toxicity of Stable Strontium in Young Pigs with the Aid of Radiostrontium. M.S. thesis, University of Illinois. 1960.

(4) Bell, M. C., Cragle, R. G., Demott, B. J., and Mraz, F. R. Semiannual progress report for January 1, 1959, to July 31, 1959. Fission Product Metabolism in Animals. U. S. AEC Doc., ORO-217: 5. 1959.

(5) Comar, C. L. Radioisotopes in Biology and Agriculture, Principles and Practice. McGraw-Hill Book Co., New York. 1955.

(6) Comar, C. L., Lotz, W. E., and Boyd, G. A. Autoradiographic Studies of Calcium, Phosphorus and Strontium Distribution in the Bones of the Growing Pig. Am. J. Anat., 90: 113. 1952.

(7) Hamilton, J. G. Decontamination Studies with Products of Nuclear Fission. Excretion of Injected Radiostrontium, Radioyttrium and Radiocerium. U. S. AEC Doc., MDDC-1001: 11. 1944.

(8) Kawin, B. Effects of Stable Calcium and Strontium on Deposition of Calcium^{45} and Strontium^{89} in Bone. Experientia, 15: 313. 1959.

(9) Kornberg, H. A. Effectiveness of Isotopic Dilution. Rad. Research, 5: 487. 1956.

(10) Kornberg, H. A. On the Passage of Pairs of Elements Through Food Chains. U. S. AEC Doc., HW-60127. 1959.

(11) Lengemann, F. W., Comar, C. L., and Wasserman, R. H. Absorption of Calcium and Strontium from Milk and Nonmilk Diets. J. Nutrition, 61: 571. 1957.

(12) LENGEMANN, F. W., WASSERMAN, R. H., AND COMAR, C. L. Enchancement of Calcium Absorption by Lactose. Federation Proc., 18: 533. 1959.

(13) MACDONALD, N. S., NUSBAUM, R. E., EZMIRLIAN, F., BARBERA, R. C., ALEXANDER, G. V., SPAIN, P., AND ROUNDS, D. E. Gastrointestinal Absorption of Ions. I. Agents Diminishing the Absorption of Strontium. J. Pharmacol. Exptl. Therap., 104: 348. 1952.

(14) MACDONALD, N. S., SPAIN, P. C., EZMIRLIAN, F., AND ROUNDS, D. E. Effects of Calcium and Phosphate in Foods on Radiostrontium Accumulation. J. Nutrition, 57: 555. 1955.

(15) MCMEEKIN, C. P. Growth and Development in the Pig with Specific Reference to Carcass Quality Character. Iowa J. Agr. Sci., 30: 276. 1940.

(16) PALMER, R. F., THOMPSON, R. C., AND KORNBERG, H. A. Effect of Calcium on Deposition of Strontium[90] and Calcium[45] in Rats. Science, 127: 1505. 1958.

(17) PALMER, R. F., THOMPSON, R. C., AND KORNBERG, H. A. Factors Affecting the Relative Deposition of Strontium and Calcium in the Rat. Science, 128: 1505. 1958.

(18) RAY, R. D., STEDMAN, D. E., WOLFF, N. K., AND LAVIOLETTE, D. Bone Metabolism. II. Toxicity and Metabolism of Radioactive Strontium (Sr^{90}) in Rats. J. Bone and Joint Surg., 38-A: 160. 1956.

(19) RUBANOVSKAYA, A. A., AND USHAKOVA, V. F. Materials on the Toxicology of Radioactive Substances. Part IV. Stimulation of the Excretion of Radioactive Strontium and Calcium from the Organism. U. S. AEC Doc., tr-3794. 229. 1959. (Original in Russian, 1957.)

(20) SCHACHTER, D., AND ROSEN, S. M. Active Transport of Ca^{45} by the Small Intestine and Its Dependence on vitamin D. Am. J. Physiol., 196: 357. 1959.

(21) SINGER, L., MAQSOOD, M., MEDLEN, A. B., AND COMAR, C. L. Endogenous and Biliary Excretion of Calcium[45] and Strontium[89]. Arch. Biochem. Biophys., 66: 404. 1957.

(22) TALMADGE, R. V., SCHOOLEY, J. C., AND COMAR, C. L. Differential Removal of Strontium[85] and Calcium[45] from Rat Skeleton by Peritoneal Lavage. Proc. Soc. Exptl. Biol. Med., 95: 413. 1957.

(23) WASSERMAN, R. H., COMAR, C. L., AND NOLD, M. M. Influence of Amino Acids and Other Organic Compounds on Gastrointestinal Absorption of Calcium[45] and Strontium[89] in the Rat. J. Nutrition, 59: 371. 1956.

(24) WASSERMAN, R. H., COMAR, C. L., AND TWARDOCK, A. R. The Secretion of Radiostrontium into Milk of the Cow and Goat: Influence of Dietary Levels of Calcium and Strontium Phosphate. J. Animal Sci., 18: 1529. 1960.

TECHNICAL NOTES

β-LACTOGLOBULIN AND pH-SENSITIVITY OF THE HEAT STABILITY OF EVAPORATED MILK [1]

Previous tests (3) on skimmilk showed that when the pH of aliquots of one sample of milk is adjusted over the range from 6.2 to 7.0 and the heat stability determined, the curve relating heat stability to pH passes through a maximum and a minimum. The occurrence of this maximum and minimum is dependent upon the presence of β-lactoglobulin (4).

Heat stability is of particular importance in the manufacture of evaporated milk. Although a complete pH curve has not been published, it is apparent (1, 2) that the heat stability of evaporated milk is influenced by pH, and it was of interest to determine if the pH-sensitivity of this product was influenced by β-lactoglobulin.

In preliminary tests, bulk or individual cow's milk was forewarmed at 95°C. for 10 min., cooled, and concentrated to half volume in a glass evaporator (5). The heat stability was then determined over a range of pH and with added calcium or phosphate. Typical results (Figure 1) indicated a maximum heat stability at pH 6.42, and a very broad minimum in the pH 6.7 to 6.9 range. Addition of phosphate increased the maximum slightly, and displaced it to the acid side, as previously observed with skimmilk (4). Addition of calcium destabilized this sample throughout the pH range studied.

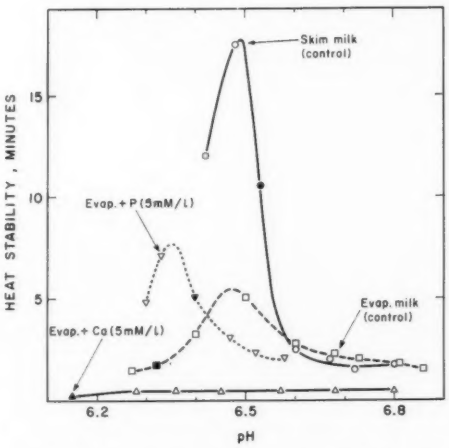


FIG. 1. Effect of pH adjustment, and of added calcium and phosphate, on the heat stability of evaporated milk. Filled points represent the samples without added acid or alkali.

Further tests were conducted with suspensions of centrifugally separated casein redispersed in ultrafiltrate, forewarmed and concentrated as outlined above. Heat stability of the control sample decreased gradually from 3 min. at pH 6.2 to 2 min. at pH 6.8. Heat stability of a suspension to which 0.3 g. of β-lactoglobulin (Nutritional Biochemicals Corp.) per 100 ml. was added before forewarming increased from 1 min. at pH 6.2 to 3.5 min. at pH 6.45, then decreased to 2 min. at pH 6.55. Similar results were obtained in a duplicate test.

Addition of β-lactoglobulin (0.3 g/100 ml.) to evaporated milk did not affect the maximum heat stability attained by pH adjustment.

These results show that evaporated milk responded to small changes in pH, and to added phosphate, in the same manner as skimmilk, and that this response was not manifested in the absence of whey protein. However, β-lactoglobulin induced much less pH-sensitivity in casein-ultrafiltrate suspensions that had been concentrated to half volume than in similar suspensions of normal casein concentration.

DYSON ROSE
Division of Applied Biology
National Research Council
Ottawa, Canada

REFERENCES

(1) BENTON, A. G., AND ALBERY, H. G. Studies on the Stability of Evaporated Milk During Sterilization with Reference to the Hydrogen Ion Concentration, Alcohol Test, and the Addition of Specific Buffers. J. Biol. Chem., 68: 251. 1926.

(2) MILLER, P. G., AND SOMMER, H. H. The Coagulation Temperature of Milk as Affected by pH, Salts, Evaporation, and Previous Heat Treatment. J. Dairy Sci., 23: 405. 1940.

(3) ROSE, D. Variations in the Heat Stability and Composition of Milk from Individual Cows During Lactation. J. Dairy Sci., 44: 430. 1961.

(4) ROSE, D. Factors Affecting the pH-Sensitivity of the Heat Stability of Milk from Individual Cows. J. Dairy Sci., 44: 1405. 1961.

(5) TESSIER, H. An Anti-Foam Low-Temperature Laboratory Evaporator. J. Biochem. Microbiol. Technol. Engng., 3: 111. 1961.

[1] Issued as N.R.C. No. 6482.

OBSERVATIONS ON THE FREE VOLATILE ACIDS IN MILK [1]

Small amounts of free volatile fatty acids in raw milk have been reported previously (1,2). This study was undertaken to determine the different free fatty acids present in milk and their possible relationship to flavor of the milk.

Forty-one fresh herd milks, collected at the time of milking, were studied. A protion of each sample was laboratory pasteurized at 145° F.—30 min., cooled, and examined for flavor. The remainder was immediately analyzed for volatile acids.

The acids were extracted from the milk by ether and the extracts were then subjected to gas chromatographic analysis (1). Usually, the acids were identified by comparing their threshold volumes with those of known acids. However, confirmation of identification was obtained by infrared analysis of fatty acids obtained from three milks. For these samples, an aliquot was analyzed by gas chromatography to establish the retention volume of the various acids. Then, fractions corresponding to the retention value of each acid were obtained from a second aliquot by trapping the gas samples of each fraction in liquid nitrogen. These samples were dissolved in carbon tetrachloride and analyzed with the Beckman IR-5 Infra-Red Spectrometer, using carbon tetrachloride as reference. The results were compared with known authentic volatile acids. Formic, acetic, butyric, valeric, caproic, and caprylic acids were identified in all three of the milk samples.

Results for both the fatty acids and the milk flavors for the 41 samples are presented in Table 1. In all, seven free fatty acids were identified as being in at least some of the samples: formic, acetic, caprylic, caproic, val-

[1] Technical Paper 361. The Department of Dairy Technology, The Ohio Agricultural Experiment Station. Ohio Agricultural Experiment Station Journal Article 7461. Supported by Hatch Funds from the U. S. Department of Agriculture.

TABLE 1

Free volatile acids in fresh raw milk of varying flavor quality

Free volatile acid	μM/50 ml. milk[a]							
	Good (13 samples)		Feed (15 samples)		Oxidized (6 samples)		Rancid (7 samples)	
	Range	Av.	Range	Av.	Range	Av.	Range	Av.
Formic	9.8–43.1	19.6	23.5–47.0	31.3	25.4–50.9	37.2	25.5–54.9	47.0
Acetic	2.6–20.5	9.0	6.4–17.9	10.2	5.1–14.1	9.0	12.8–28.1	16.6
Propionic	0–1.5	0.3	0–1.9	0.6	0–0.4	0.2	0–1.5	0.5
Butyric	0.1–1.9	0.4	0.3–1.8	0.8	0–.7	0.3	4–10.9	7.6
Valeric	0–1.9	0.7	0.1–1.3	0.6	0–0.9	0.4	0.9–1.7	1.2
Caproic	2.3–4.6	3.0	1.7–4.6	3.0	1.9–40	2.9	5.7–8.0	6.9
Caprylic	4.8–7.4	6.7	5.9–14.8	8.9	4.6–6.4	5.3	7.2–12.5	9.6

[a] Values corrected to 100% recovery.

TABLE 2

Changes in free volatile free fatty acids in eight fresh and stored pasteurized milks

Analysis	μM/50 ml. milk[a]							
	Stored at 40° F.							
	Fresh		7 Days		14 Days		21 Days	
	Range	Av.	Range	Av.	Range	Av.	Range	Av.
Flavor score[b]	37.5–38.5	38	37.5–38	38	36–38	37	Samples	Spoiled
Bacterial count × 10[3]	7–46	23	21–76	47	56–3,300	238	2,600–66,500	23,400
Free vol. acids	3.3–23.5	15.9	1.9–27.6	12.9	4.3–46.2	17.7	103–380	160
Formic								
Acetic	2.6–7.7	6.8	2.8–7.6	5.7	2.6–22.6	9.6	11–199	95
Propionic	0.1–0.6	0.2	0.1–0.4	0.2	0.1–0.5	0.2	0.7–2.7	0.5
Butyric	0.7–1.2	0.9	0.6–1.1	0.8	0.8–2.4	1.2	2.4–9.6	5.6
Valeric	0–0.4	0.1	0.1–0.5	0.2	0.1–0.6	0.3	0.7–2.7	1.5
Caproic	0.3–13.6	0.7	0.3–14.7	0.8	0.4–17.6	1.0	2.2–8.9	6.0
Caprylic	0.6–1.6	0.8	0.6–1.4	0.8	0.6–1.7	0.8	0.8–10.6	5.6

[a] Values corrected to 100% recovery. All values based on increase in concentration as compared to fresh raw milk.

[b] Averages computed to nearest whole number.

eric, butyric, and propionic. Only the first four acids were found in all milks. For good-flavored milk, the relative concentrations of the acids were in the order in which they are listed.

Of the 41 milks, 13 were considered free of off-flavors, 15 had feed flavors, six were oxidized, and seven were rancid. Milk with feed flavors usualy contained relatively higher average concentrations of formic, butyric, and caprylic acids; whereas, for rancid-flavored milk, butyric, caproic, caprylic, formic, and acetic acids were present in comparatively high levels. The milk with oxidized flavor was not characterized by a characteristic fatty acid pattern.

In an additional experiment, eight paired lots of raw and pasteurized milk were obtained from commercial companies. The raw samples were obtained from the system just before the milk passed into the pasteurizer; the pasteurized samples were obtained directly from the bottle filler. Pasteurization temperatures varied from 170 to 180°F. and holding times from 16 to 22 sec. The raw and pasteurized milks were analyzed immediately for flavor, volatile acids, and total bacterial counts. The pasteurized samples were stored at 40°F. and evaluated for flavor, analyzed for fatty acids, and standard plate counts made after seven, 14, and 21 days of storage. Table 2 presents the results.

The data reveal that commercial pasteurization produced a general increase in the free fatty acids, but the increase was significant only for the formic and acetic acids: averaging 16.9 μM/50 ml. and 6.8 μM/50 ml., respectively. The heat treatments used generally caused a slight heated flavor. The pasteurized milk showed no significant changes in flavor, bacterial and free fatty acid during the first seven days of storage. After 14 days, bacterial counts had increased and the concentration of free fatty acids increased slightly. The sample with the 3.3 million bacterial count contained the highest concentration of all free fatty acids. After 21 days, the samples were spoiled and total bacterial numbers exceeded 2.6 million in all samples. Very substantial increases in free fatty acids accompanied the spoilage, with formic and acetic acid values increasing about ten times in concentration as compared to the 14-day samples. Approximately fivefold increases were observed for butyric, valeric, caproic, and caprylic acids.

W. J. HARPER
I. A. GOULD
AND
C. L. HANKINSON
Department of Dairy Technology
The Ohio Agricultural Experiment Station
Columbus

REFERENCES

(1) HANKINSON, C. L., HARPER, W. J., AND MIKOLAJCIK, E. A Gas-Liquid Chromatographic Method for Fatty Acids in Milk. J. Dairy Sci., 41: 1502. 1958.
(2) MORR, C. V., HARPER, W. J., AND GOULD, I. A. Acids Produced by High Heat Treatment of Milk. J. Dairy Sci., 40: 964. 1957.

FURACIN RESIDUES IN MILK[1]

Furacin[2] is a preparation for the treatment of bovine mastitis which is available only at the professional level. It is being used by veterinarians in cases where prior treatment by the milk producer with other mastitis preparations has failed. The manufacturers advise that milk from treated quarters of udders should be discarded for 72 hr. after the last treatment if the Furacin preparation also contains penicillin. It is recommended that the milk from quarters treated with Furacin alone need be discarded for only 24 hr. after the last treatment (1). Furacin is effective against *Bacillus subtilis* (3), the microorganism commonly used in the disc assay method for the detection of penicillin and other antibiotic residues in milk. Its effect upon growth and scouring in dairy calves has been evaluated (2).

This investigation was undertaken for the purpose of determining the minimum level of Furacin detectable in milk using the disc assay (4) and the period of time the milk should be discarded after the last treatment with Furacin alone.

Samples of raw milk were prepared containing concentrations of Furacin ranging from 0.2 mg/ml to 0.03 μg/ml. Another set of samples containing similar Furacin concentrations was prepared in distilled water. Duplicates of all samples in both milk and water were heated to a minimum temperature of 85° C. for 5 min. before assay to avoid the possibility of false readings.

Results show that heating Furacin in milk or water did not affect the diameter of the zones produced. Other results, presented in Table 1, show that 0.03 μg. of Furacin (the lowest level tested) could be detected using the disc assay method. It should be noted that the zones produced had uniformly small diameters, with little difference between the 0.03 μg/ml and 25 μg/ml levels of Furacin. The zone produced by 200 μg/ml of Furacin was of the same diameter as that produced by 0.1

[1] Technical contribution No. 369, South Carolina Agricultural Experiment Station, Clemson Agricultural College, Clemson, South Carolina.

[2] Trade name for 5 nitro, 2 furaldehyde semicarbazone, and a product of Eaton Laboratories, Norwich, New York.

TABLE 1

Diameter of zones produced by Furacin with disc assay using *Bacillus subtilis*

Sample	Conc.	Zone diameter
	(μg/ml)	*(mm.)*
Penicillin	0.05*	14.0
	0.10*	16.0
	5.00*	22.0
Blank milk disc (disc diameter)	0.00	7.0
Furacin	200.00	14.5
	100.00	12.5
	50.00	10.0
	25.00	9.0
	12.50	8.0
	6.25	8.0
	3.12	8.0
	1.56	8.0
	0.78	8.0
	0.39	8.0
	0.19	8.0
	0.09	8.0
	0.05	8.0
	0.03	7.5

* Penicillin concentration in units/ml.

unit/ml of penicillin, one of the penicillin levels used to check the sensitivity of the assay.

Because zones produced by Furacin were smaller than those produced with comparable levels of penicillin, duplicate assays were made on all samples. One set of duplicates was refrigerated at 2° C. for 18 hr., to gain more time for Furacin diffusion, in hopes that this technique would produce zones of greater diameter from a given level of Furacin. No increase in zone diameter occurred. All samples were assayed on both Whey and Penn Assay agars. Zone diameters were the same on both media, but the Penn Assay agar zones were more clear, a distinct advantage with the small-diameter zones produced. The circumference of each Furacin-produced zone showed a ring of stimulated growth similar to the rings produced by penicillin. Furacin-produced zones were of equal diameter in both milk and water.

Eight dairy cows of assorted breeds were infused with 30 ml. of a 0.2% Furacin preparation in one quarter. The infused quarters were milked 24, 48, 72, and 96 hr. post-infusion, using a quarter milker. The entire milking from the quarter was then sampled and assayed. No trace of Furacin was found in any sample and it appears that no active Furacin residue can be found in the milk from treated quarters 24 hr. after the last treatment. Unpublished data indicate that Furacin is inactivated in vitro when exerting antibacterial activity (1). The same data also indicate that Furacin is inactivated by tissues.

SUMMARY

No Furacin residues were found in milk from treated quarters 24 hr. after the last treatment. Levels of Furacin as low as 0.03 μg/ml were detected with the disc assay method against *B. subtilis*, in milks to which known concentrations of Furacin had been added. The zones were clear-cut, distinct, but much smaller than those produced by comparable levels of penicillin.

R. W. Henningson
Dairy Department, Clemson College
Clemson, S. C.

REFERENCES

(1) Andrews, M. F. Personal correspondence, Letter, January 19. Norwich, N. Y. 1961.
(2) Gardner, K. E., and Wittorff, W. E. The Effect of Feeding Nitrofurans upon the Growth of Dairy Calves. J. Animal Sci., 14: 1204. 1955.
(3) Martin, J. E. Characteristics and Uses of Nitrofurans in Veterinary Medicine. Proc. 1st Regional Conf. Nitrofurans in Vet. Med. p. 37. Wilmington, Delaware. 1958.
(4) Silverman, G. J., and Kosikowski, F. V. Systematic Testing of Inhibitory Substances in Milk. J. Milk and Food Technol., 15: 120. 1952.

TRACHEAL TUBE FOR THE TOTAL COLLECTION OF RUMEN AND RESPIRATORY GASES

Tracheostomized animals have been used for nutritional and physiological studies periodically since the late Nineteenth Century. To make energy calculations using the respiratory-quotient method, it is necessary to correct carbon dioxide measurements for any carbon dioxide produced from rumen fermentation. For energy calculations using the carbon-nitrogen balance method it is necessary to make a complete collection of methane and carbon dioxide.

The data of Klein (9) suggest that approximately one-third of the methane formed in the intestinal tract was absorbed and exhaled through the lungs when tracheal and chamber collections were compared. Cresswell's (4) study on methane excretion through the lungs, which related methane absorption to oxygen consumption, might indicate that the absorption was less extensive. Preliminary calculations on our own work involving simultaneous comparisons of tracheal collection of expired gas and chamber collection of total gas from cattle support a magnitude of absorption suggested by the data of Klein (9).

Carbon dioxide absorption from the rumen can not be measured directly as can the absorption of methane, since carbon dioxide is produced from both rumen and tissue metabo-

lism. That absorption of carbon dioxide from the rumen can occur is evident from the work of Dougherty (6), which showed an increase in portal vein carbon dioxide after rumen insufflation with carbon dioxide. Blaxter (1) reasoned that the absorption of carbon dioxide from the rumen is more rapid than the absorption of methane. This more rapid diffusion of carbon dioxide has been found by many workers studying tissues other than rumen tissue. It is evidenced by the studies of Teschendorf (12) on gas diffusion through rabbit peritoneum and the studies of McIver *et al.* (11) on gas diffusion through cat small intestine. Further support for this concept is the work of Zuntz *et al.* (13), which showed that the ratio of carbon dioxide to methane found during in vitro fermentation is higher than that found in vivo. Also, the work of Lehmann *et al.* (10) showed a higher ratio of carbon dioxide to methane in intestinal gas of recently killed animals compared to that caught from the living animal.

Thus, collection of respiratory gas from the posterior trachea alone does not give gas exchange from tissue metabolism uncontaminated with rumen gases. Secondly, on collection from the posterior trachea alone, a part of the rumen gases escape by eructation and are not available for making a complete carbon balance. Therefore, it appeared desirable to measure the entire gas exchange. Colvin *et al.* (2), Cresswell (4), and Dougherty (7) have shown that eructated gas flowed down the trachea. This would indicate that it might be possible to get the total gas exchange by connecting only to the trachea. Suitable connecting tubes have been devised using a simple dipping mold, a commercially available plastic,[1] and a laboratory muffle furnace for curing.

Molds were made in a machine shop,[2] using round steel stock of appropriate size (see Figure 1). For 800- to 1,000-lb. cows this size was 1 in. The right-angle joints are cut on a radius and held together by screws through the base. To make a tube, the mold is preheated in a muffle furnace, and is then inverted and dipped into the plastic. The gap between the two sections of the mold fills with plastic to form a diaphragm between the upper and lower ends of the tube. Tube thickness is increased by increasing mold preheat temperatures or by increasing the dipping time within the ranges suggested by the manufacturer. The coated mold is then removed from the liquid plastic and rotated to prevent any flowing on the mold. After flowing stops, the mold is cured in a muffle furnace. After cooling, the mold can be removed from the tube. To do this, the ends of the tube are cut away and the screws

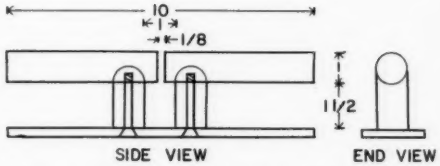


FIG. 1. Tracheal tube mold. Dimensions are in inches.

are removed. Glycerol will aid in this process.

To form a seal in the trachea, the inflatable single-wall cuffs used earlier (8) may be used, but they tend to narrow the lumen of the soft plastic tubes described here. It appears more desirable to have a thin-wall tube which has less restriction on air flow through the trachea. If tubes of appropriate size are used, tracheal secretions form an adequate seal.

The tube is used for collection from animals prepared as outlined in an earlier publication (8). It makes two separate connections to the trachea available. Eructated rumen gas is collected through a one-way valve attached to the anterior opening. Expired gas from the lungs is collected from the posterior opening, using a suitable two-way valve. These collections may be done simultaneously and kept separate. A mixed collection is made by connecting the anterior opening to the exhaust side of a two-way valve attached to the posterior opening.

Based on preliminary calculations using methane and carbon dioxide ratios, nearly all of the methane is collected when a simultaneous collection is made from both ends of the trachea. Further evidence of total collection from the trachea is the finding of Conrad *et al.* (3), that an infrared carbon dioxide analyzer showed no carbon dioxide increase in mask air at the time of eructation. Thus, it appears that all eructated rumen gas makes a passage into the lungs before leaving the animals.

Gases from the lungs are partially contaminated with absorbed rumen gases. Eructated rumen gases can be collected from the upper end of the trachea. A tracheal tube allowing the collection of gas from both ends of the trachea offers possible advantages over the type described by Cresswell and Harris (5) by allowing tracheotomized animals to be used for carbon-nitrogen balance experiments or by using the same corrections as used for chambers in respiratory-quotient calculations of heat production.

D. R. WALDO
H. HOERNICKE[3]
Dairy Cattle Research Branch
Animal Husbandry Research Division, ARS
Agricultural Research Center
Beltsville, Maryland

[1] Plastisol Series 64, Chamberlain Engineering Corporation, P. O. Box 350, Akron 9, Ohio.

[2] Instrument Shop, Soil and Water Conservation Research Branch, Plant Industry Station, Beltsville, Maryland.

[3] Recipient of a Deutsche Forschungsgemeinschaft Fellowship and a Fulbright Travel Grant.

REFERENCES

(1) Blaxter, K. L. Ruminant Nutrition. *In* Progress in the Physiology of Farm Animals. Ed., J. Hammond. Vol. 1, p. 26. Butterworths Scientific Publications, London. 1954.

(2) Colvin, H. W., Jr., Wheat, J. D., Rhode, E. A., and Boda, J. M. Technique for Measuring Eructated Gas in Cattle. J. Dairy Sci., 40: 492. 1957.

(3) Conrad, H., Engelhardt, W. v., and Hoernicke, H. In preparation.

(4) Cresswell, E. Methane Exhalation in Sheep. Nature, 186: 560. 1960.

(5) Cresswell, E., and Harris, L. E. An Improved Trachael Cannula for Use in Indirect Calorimetry and Other Studies. Vet. Record, 73: 343. 1961.

(6) Dougherty, R. W. Physiological Studies of Induced and Natural Bloat in Dairy Cattle. J. Am. Vet. Med. Assoc., 96: 43. 1940.

(7) Dougherty, R. W. The Physiology of Eructation in Ruminants. *In* Digestive Physiology and Nutrition of the Ruminant. Ed. D. Lewis. p. 84. Butterworths Scientific Publications, London. 1961.

(8) Flatt, W. P., Waldo, D. R., Sykes, J. F., and Moore, L. A. A Proposed Method of Indirect Calorimetry for Energy Metabolism Studies with Large Animals Under Field Conditions. Proc. First Symposium on Energy Metabolism, Copenhagen. p. 101. September, 1958.

(9) Klein, W. Zur Ernaehrungsphysiologie landwirtschaftlicher Nutztiere, besonders des Rindes. Biochem. Z., 72: 169. 1915.

(10) Lehmann, F., Hagemann, O., and Zuntz, N. Zur Kenntnis des Stoffwechsels beim Pferde. Landwirtsch. Jahrb., 23: 125. 1894.

(11) McIver, M. A., Redfield, A. C., and Benedict, E. B. Gaseous Exchange Between the Blood and the Lumen of the Stomach and Intestines. Am. J. Physiol., 76: 92. 1926.

(12) Teschendorf, W. Ueber die Resorptionszeit von Gasen in der Bauchhoehle. Arch. exptl. Pathol. Pharmakol., 92: 302. 1922.

(13) Zuntz, N., v.d. Heide, R., Klein, W., v. Markoff, J., v. Dschandieri, —., and Diakow, —. Zum Studium der Respiration and des Stoffwechsels der Wiederkaeuer. Landwirtsch. Vers.-Sta., 79/80: 781. 1913.

VOLATILE FATTY ACID ANALYSES OF BLOOD AND RUMEN FLUID BY GAS CHROMATOGRAPHY

Short-chain fatty acids that originate from fermentation by rumen microflora are absorbed through the rumen wall and provide the principal source of energy for the ruminant animal. Furthermore, the relative concentrations of these fatty acids have been related to efficient energy utilization (2) and to milk fat production (5) in dairy cattle. Consequently, the development of an accurate, rapid method of short-chain acid analyses in blood and rumen fluid is of primary importance in ruminant nutrition. The recovery of radioactive acetate and butyrate was used in developing a suitable method for the preparation of rumen fluid and blood. Direct analyses of the resulting supernatant for short-chain acids were performed by gas chromatography.

In preliminary studies, rumen fluid, in which microbial activity had been destroyed, was acidified and extracted with diethyl ether for 20 hr. Initially, sodium methylate was added to the receiving flash containing ether and, following extraction, the sodium salts of fatty acids were acidified and diluted to volume. Only 81.4 and 74.9% of the radioactive acetate were recovered in duplicate samples; the remaining radioactivity was found in the extracted rumen fluid and in the ether layer which remained in the extraction bulb. With the same procedure, the recovery of radioactive butyrate was 96.2% whereas, again, acetate was only 78.4%.

Since acetate could not be completely removed by liquid-liquid extraction with ether, an alternate method of preparing rumen samples was adopted. With this alternate method, samples of rumen fluid were treated with metaphosphoric acid and centrifuged at 3,000 r.p.m. for 10 min. The recoveries of radioactive acetate in the supernatants of the duplicate samples were 91.0 and 90.5%.

As with rumen fluid, the recovery of radioactive acetate varied between 41.0 and 57.0% in whole blood that was continuously extracted with diethyl ether for 24 hr. With the use of either sulfosalicylic acid or sulfuric acid-sodium tungstate as protein precipitant, the recoveries of acetate in the supernatants were 91.5 and 92.2%. Since the short-chain acids are present in low levels in blood, it was necessary to concentrate the protein-free filtrate by lyophilization. Complete recovery of acetate was verified by the use of radioactive acetate.

Most existing methods for short-chain fatty acid analyses that employ steam distillation and/or partition chromatography lack sensitivity and do not separate the isoacids accurately. Several methods involving gas-liquid chromatographic procedures have been used by various investigators (1, 3) since the initial development of a method by James and Martin (6). However, these gas chromatographic procedures for short-chain volatile fatty acids require extensive sample preparation which involves steam distillation, formation of sodium salts, evaporation, and/or conversion to the methyl esters.

Previously, Emery and Koerner (4) reported a method of separating trace amounts of short-chain fatty acids in water with the use of the flame ionization detector and a column packing that contained Tween 80 as the liquid phase. In water, the free acids were eluted as sym-

metrical peaks (ether solutions of free acids caused excessive tailing of the peaks) and over 100 aqueous samples were successfully analyzed by this column mixture. However, with several other sources of Tween 80, the column performance could not be duplicated and excessive peak tailing, variable response to the various acids, and ghosting were observed. (The term ghosting refers to the appearance of peaks, with the same retention time as the various fatty acids, when distilled water is injected.) Originally, it was thought that this was due to contamination by a previous sample, but the same ghosting effect was observed following a thorough cleaning of the entire system. Consequently, the Tween 80 must retain some fatty acids in the column and an injection of water elutes them in proper order and retention time.

Metcalfe (7) utilized phosphoric acid in his polyester column packing to overcome the association effect of fatty acids and consequently to develop more symmetry in eluted fatty acid peaks. Free fatty acids (acetic to valeric) in ether were separated with symmetrical peaks when Metcalfe's column mixture was used. However, the isoacids could not be separated from the next lower carbon normal acid. When phosphoric acid was added to Tween 80 as the liquid phase on Chromosorb W, sharp symmetrical peaks from free fatty acids and their isomers were resolved (Figure 1). With this column a variety of solvents (water, methanol, and ether) can be used for short-chain acids, although the use of water or methanol results in ghosting. To test the effect of ghosting on analytical error, four aqueous solutions of short-chain acids and their isomers from acetate to valerate of different concentrations (1.0, 0.5, 0.1, and 0.05%) were analyzed in varying order in a complete randomized Latin-square design. Even though some fatty acids are eluted when distilled water is injected into the column following a fatty acid mixture, the sequence of analyses of the fatty acid solutions did not significantly alter the precision of the method. Apparently, an equilibrium exists between the fatty acids retained and those moving through the column. Over the wide range of fatty acid concentration from 0.05 to 1.0%, the detector response does not appear linear in the 0.05 to 0.10% range. These differences include those errors encountered in dilution (Table 1), as well as those in measuring 0.02 ml. for injection. Consequently, each day a standard solution that contains a fatty acid concentration similar to the biological samples should be analyzed. An integration of peak area was compared to peak height as a measure of fatty acid concentration. Since the various fatty acids produce sharp symmetrical peaks, peak height can be used with precision similar to that obtained with the Disc Integrator (Table 1). Greater precision can be obtained by increasing peak height through reduction of attenuation. The Flame Ionization Detector is extremely sensitive and, in aqueous solutions, 0.01 μg. of acetic acid resulted in a mean peak height of 280 mm.

Six replicates of a rumen sample withdrawn from a sheep fed a diet that contained 91% alfalfa hay, 8% molasses, and minerals were analyzed for volatile fatty acids to determine reproducibility of the method. The μmol/ml and the respective standard deviations of the fatty acids were as follows: acetate, 62.1 $\pm$ 0.3; propionate, 16.9 $\pm$ 0.2; butyrate, 10.6 $\pm$ 0.1; valerate, 1.2 $\pm$ 0.01. Blood was withdrawn from the jugular vein of a sheep fed an all-concentrate diet. Reproducibility of the blood fatty acid method was estimated from seven replicate analyses. The following mM/L whole blood and

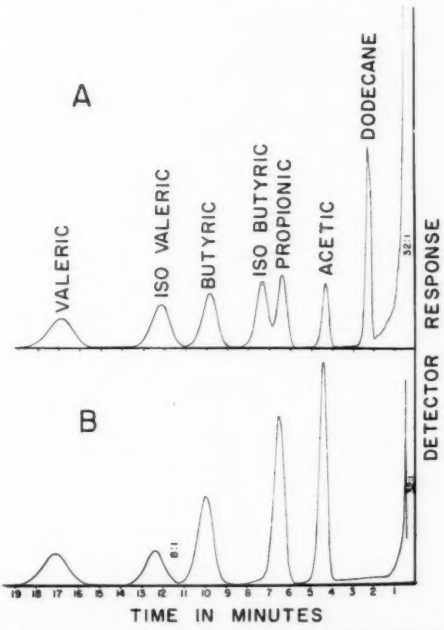


FIG. 1. A = Separation of short-chain acids in 0.1% ether solution with 0.05% dodecane.
B = Separation of short-chain acids in aqueous rumen fluid. In each case, 0.02 ml. was injected into the column.

TABLE 1

Per cent recovery from aqueous solutions of fatty acids varying in concentration

Per cent concentration	Acetate		Valerate	
	Counts[a]	Peak height	Counts[a]	Peak height
1.0	100.0	100.0	100.0	100.0
0.5	96.6	98.0	94.7	97.7
0.1	98.2	94.2	96.1	97.7
0.05	90.3	89.1	101.1	101.5

[a] From Disc Integrator (Disc Instruments, Inc., Santa Ana, California).

the respective standard deviations were found: acetate, 2.431 ± 0.032; propionate, 0.092 ± 0.004; butyrate, 0.45 ± 0.002. These amounts and ratios of volatile fatty acids in the blood and rumen samples are similar to those previously reported for animals fed similar diets.

Lactic and pyruvic acids are important constituents of many biological systems. However, they can not be eluted per se from this column. Following treatment with diazomethane, lactic acid (as the methyl ester–methyl ether) and pyruvic acid (as the methyl ester ketone) can be readily eluted and accurately determined. The polarity of this column adds to the versatility of analyses of organic materials that can be separated with this column mixture. In an attempt to find a suitable marker for the free fatty acids, the following organic compounds have been separated with this column:

1. Normal and branched paraffins and olefins
2. Primary, secondary, and tertiary alcohols
3. Methyl esters of fatty acids from acetate to stearate
4. Various functional groups such as alcohols, olefins, alkanes, acids, and aldehydes
5. Free acids from acetic to palmitic

When ether or methanol is used as a solvent, dodecane can be used as a marker to make the analyses quantitative, since its retention time is slightly less than that of acetate (Figure 1A).

SUMMARY OF A METHOD FOR THE DETERMINATION OF SHORT-CHAIN ACIDS IN RUMEN FLUID AND BLOOD

Rumen fluid. Into a 15-ml. centrifuge tube are added 5 ml. of strained rumen fluid and 1 ml. of metaphosphoric acid (25%). After standing for 30 min., the contents are centrifuged at 3,000 r.p.m. for 10 min. The supernatant can be analyzed by gas chromatography without further preparation.

Blood. To 20 ml. of blood are added 80 ml. 0.2 *N* H_2SO_4. After 10 min., 20 ml. of 10% Na tungstate are added and the contents filtered. The filtrate is lyophilized after the sodium salts of the fatty acids are formed by the addition of 1 ml. of 3 *N* NaOH to 50 ml. of the protein-free filtrate. The salts are acidified with 2 ml. of 1.6 *N* H_2SO_4 or with acidified ether or methanol. Methanol or ether is preferred, since dodecane can be used as a marker to quantitate the determination. (The retention time of dodecane is just less than that of acetic acid.) The solutions can be directly used without further preparation. Although sulfosalicylic acid can be used as the protein precipitant, sometimes it is degraded on the column resulting in several peaks, one with the same retention time as phenol and others with the same retention time as some of the short-chain acids.

Gas chromatographic method. The instrument used was a Perkin-Elmer Model 154-C Vapor Fractometer fitted with a Flame Ionization Accessory Kit. The injection block was maintained at 250° C. to provide rapid vaporization of sample. The column was packed with 20% Tween 80, 2% phosphoric acid (85%) on Chromosorb W. Both 1- and 2-meter ¼-in. stainless steel columns were tried. Since the longer column showed no advantage in peak sharpness or in fatty acid separation, the 1-meter column was selected. A column temperature of 120° C. was used with a nitrogen carrier gas flow rate of 120 ml/min. The hydrogen flow to the flame jet was 35 ml/min and air flow to the detector chamber was 250 ml/min. A Perkin-Elmer No. 0 restriction needle was attached to the by-pass line to alternate the flow to the detector. A direct current potential of 300 volts was used across the flame. The output of the ionization detector amplifier was transmitted to a 5-mv. recorder. Under these conditions, free fatty acids and their isomers can be eluted as symmetrical peaks in 18 min. Figure 1 illustrates the separation of the short-chain free fatty acids obtained by this method with a standard mixture in ether and with rumen fluid.

In aqueous solutions, the amounts of short-chain acids can be obtained by determining peak areas of the acids in a standard solution and comparing them to the unknown. A marker such as dodecane is preferred when volatile solvents such as ether or methanol are used. Comparative peak areas of dodecane and fatty acids in known concentrations are first established. By accurately adding a known aount of dodecane to unknown samples, quantitative amounts of fatty acids can be determined by adjusting the fatty acid peak areas to the area of dodecane.

E. S. Erwin
G. J. Marco
E. M. Emery
Monsanto Chemical Company
St. Louis, Missouri

REFERENCES

(1) Annison, E. F. Studies on the Volatile Fatty Acids of Sheep Blood with Special Reference to Formic Acid. Biochem. J., 58: 670. 1954.

(2) Armstrong, D. G., and Blaxter, K.L. Utilization of Acetic, Propionic, and Butyric Acids by Fattening Sheep. British J. Nutrition, 11: 413. 1957.

(3) Bensadoun, A. Direct Determination of the Absorption of Volatile Fatty Acids from the Gastrointestinal Tract of Ruminants. Part I. Short Chain Volatile Fatty Acid Analysis in Biological Materials. Ph.D. dissertation, Cornell University, Ithaca, N.Y. 1960.

(4) Emery, E. M., and Koerner, W. E. The Gas Chromographic Determination of Trace Amounts of Lower Fatty Acids in Water. Anal. Chem., 33: 146. 1961.

(5) Ensor, W. L., Shaw, J. C., and Tellechee, H. F. Special Diets for the Production of Low Fat Milk and More Efficient Gains in Body Weight. J. Dairy Sci., 42: 189. 1959.

(6) JAMES, A. T., AND MARTIN, A. J. P. Gas-Liquid Partition Chromatography: The Separation and Micro-estimation of Volatile Fatty Acids from Formic to Dodecanoic Acid. Biochem. J., 50: 679. 1952.
(7) METCALFE, L. D. Gas Chromatography of Unesterified Fatty Acids Using Poly-ester Columns Treated with Phosphoric Acid. Nature, 188: 142. 1961.
(8) VORBECK, M. L., MATTICK, L. R., LEE, F. A., AND PEDERSON, C. S. Determination of Fatty Acids of Lower Molecular Weight by Gas Chromatography. Nature, 187: 689. 1960.

EXPERIMENTAL PRODUCTION OF BLOAT WITH VARIOUS CHEMICALS [1, 2]

Bloat is a malady of ruminants characterized by the inability of the animals to expel rumen gases. Variations in susceptibility to bloat and in the severity of bloat among and within animals suggest that this variance represents varying degrees of inhibition of eructation. It may be assumed that eructation is controlled primarily by the autonomic nervous system. From a physiological standpoint, bloat may occur because of the inability of the animal to eructate during a certain period, thus trapping the gases continually being produced by the rumen fermentation. Agents which control the autonomic nervous system should cause bloat when they effectively inhibit eructation; conversely, they may relieve bloat if inhibition of eructation is overcome. This report deals with experimentation aimed at the more probable physiological mechanisms which may be involved in bloat.

Intramuscular injections of epinephrine to stimulate the sympathetic and of atropine to inhibit the parasympathetic nervous systems were administered to ruminants. Except for one Jersey steer on bluegrass pasture and one goat fed alfalfa hay in dry lot, the experimental animals were sheep and lambs ranging from 70-150 lb. in weight, which had access to bluegrass pasture and were allowed ad libitum consumption of alfalfa hay. The animals had no history of bloat on these diets. Injections were made until bloat occurred, usually within an hour, or for from 2 to 3 hr. in animals which showed no bloat. Atropine injections were made on an hourly basis and epinephrine was injected at 15- to 30-min. intervals. Due to variations in animal size and treatment time, drug dosages are reported in terms of mg/kg/hr. Results of bloat observations on sheep injected with atropine and epinephrine are given in Table 1.

It will be noted that high levels of either drug alone resulted in only slight tympany, although large doses of epinephrine resulted in hypersensitivity, hyperpnea, and frequent urination, symptoms often often associated with acute bloat (1). The steer was twice injected with dosages of .09 mg/kg/hr of epinephrine and 1.93 mg/kg/hr of atropine and bloated each time. The goat did not bloat at drug levels effective in the sheep, but was bloated with a dosage of .24 mg/kg/hr of epinephrine and 7.0 mg/kg/hr of atropine.

To investigate the possibility of a linkage between the production of bloat with epinephrine and atropine and the natural phenomenon, sheep were fasted for 15 hr., then allowed to graze green alfalfa for 2.5 hr., in attempts to produce natural bloat. Three animals which showed slight distention of the rumen after such grazing were injected with small amounts of epinephrine (.05 mg/kg) and atropine (.70 mg/kg); two animals which exhibited no bloat after grazing were similarly injected. In the first three sheep bloat increased to severe stages and receded within 2 hr. In the two animals which showed no distention after grazing, one bloated moderately, whereas the other did not bloat after in-

[1] Scientific Article No. A899, Contribution No. 3224 of the Maryland Agricultural Experiment Station.

[2] This work was supported in part by Animal Disease and Parasite Research Division, ARS, USDA.

TABLE 1

Incidence and severity of bloat in sheep injected with epinephrine and atropine

Dosage, mg/kg/hr		Animals injected	Bloat cases			
Epinephrine	Atropine		None	Slight	Moderate	Severe
	2.00– 4.00	4	4			
	4.00– 7.00	5	1	3	1	
	7.00–10.00	2		2		
0.17–0.23		5	5			
0.70–0.74		2		2		
0.03–0.08	0.40– 0.80	8	8			
0.09–0.14	0.87– 1.20	21	4	3	8	6
0.09–0.14	1.21– 1.93	15	1	7	5	2
0.15–0.19	1.21– 1.93	11		7		4
		73	23	24	14	12

jection. These quantities of epinephrine and atropine were not sufficient to bloat sheep fed on bluegrass pasture or alfalfa hay in dry lot. The possible linkage between natural and experimental bloat is supported by the demonstration of an additive effect; an effect which may be expected only when the mechanism concerned is the same or when the causative factors have influenced the same physiological sites either directly or indirectly.

Feedstuffs associated with bloat, such as green legumes, may contain substances which either by themselves or as metabolic products of the rumen micro-organisms affect the autonomic nervous system. Orally administered tyrosine (1-5 g. in the sheep, quantities that could be consumed in green legumes) replaced epinephrine when used in conjunction with atropine in the experimental production of bloat. Varying intensities of bloat were produced in 17 cases (16 in sheep, one in cattle) with doses ranging from 0.02 g/kg to 0.22 g/kg of L-tyrosine, or from 0.06 g/kg to 0.4 g/kg of DL-tyrosine. These dosages indicate that L-tyrosine was the active form. Tyrosine, in acid fermentations, is readily decarboxylated to form tyramine which is sympathomimetic. Normally, the pH of the rumen contents is in the acid range after the ingestion of food. Thus, tyrosine may be converted to tyramine, which accounts for its observed activity in the experimental production of bloat. These results also suggest that precursors of sympathomimetic compounds in feedstuffs may be instrumental in naturally occurring bloat.

Alkaloids are known to exist in legumes (3). Some of these alkaloids are parasympatholytic. Following procedures outlined by Hamerslag (2), crude alkaloid extracts were prepared from green alfalfa and Ladino clover. These extracts, in quantities derived from 5 lb. of green legume, were found to have bloat-producing activity when orally administered with tyrosine to the sheep. Feeding of the initial methanol extract of green alfalfa resulted in more severe bloat than that observed with the alkaloid extracts, suggesting that other components of alfalfa may be of importance in the bloat syndrome.

The ruminant animal has a mechanism which when activated will release the accumulated rumen fermentation gases. This mechanism must respond to intraruminal pressures and it can be expected that there are neuro-pressure receptors in the rumen linked to or a part of the autonomic nervous system. There is a pressure threshold above which eructation will occur. Inhibition of the mechanism raises this threshold and variation in inhibition could account for variations in severity of bloat.

The large variation in conditions which can result in bloat is due to the many interrelated variables which influence it. The considerable difference in animal responses to compounds which affect the nervous system, variations in consumption of a particular bloat-producing diet within a certain period, and variations in composition of particular plant materials may all influence the incidence of bloat.

E. C. Leffel
R. J. Komarek
Department of Animal Husbandry
University of Maryland
College Park

REFERENCES

(1) Cole, H. H., Dougherty, R. W., Huffman, C. F., Hungate, R. E., Kleiber, Max, and Maclay, W. D. A Review of Bloat in Ruminants. Natl. Research Council, Publ. 388. 1956.
(2) Hamerslag, F. E. The Technology & Chemistry of Alkaloids. D. Van Nostrand Co., Inc., N. Y. 1950.
(3) White, E. P. Alkaloids of the Leguminosae. I-VII. Part I, A Survey of Legumes Examined for Alkaloids. New Zealand J. Sci. Technol., 25:93. 1943.

GROWTH STIMULATION OF *BUTYRIVIBRIO* BY MUCIN

In most work involving the culture of rumen bacteria, rumen fluid has been incorporated in the media to stimulate growth, and has been found to be essential for growth of some of these organisms (1–5, 9). Several components of ruminal ingesta have been found to provide stimulation or fulfill requirements for certain rumen organisms or to stimulate cellulose digestion by cultures of rumen organisms. The active compounds include valine, proline, leucine, and isoleucine (7), branched and straight-chain fatty acids (5, 6), and several less well identified compounds. Gill and King (8) demonstrated that rumen fluid stimulated the growth of *Butyrivibrio* cultures in defined media.

In the present study, growth of a culture of *Butyrivibrio fibrisolvens* (different from the strain used by Gill and King) was stimulated by sterile rumen fluid which was added to a basal medium containing mineral salts, an amino-acid mixture, glucose, and a vitamin mixture, similar to the medium of Gill and King (8). The amount of growth stimulation was determined after 22 hr. of incubation in matched tubes by measurement of turbidity in a Klett-Summerson colorimeter with a No. 66 red filter. Fractionation of the rumen fluid indicated that the active ingredient(s) was/were organic in nature (destroyed by ashing), nondialyzable, not extracted by lipid solvents, and stable to autoclaving at 121°C. for 15 min. The activity was partially retained on anion and cation exchange resins, and could be eluted with strong acids. The presence of nitrogen, but absence of activity in amino acid fractions, indicated that the active material might be mucopolysaccharide.

Granular hog mucin (Type 1701 W; Wilson Laboratory, Chicago, Illinois.) at concentrations

of 0.5, 1.0, and 2.0 mg/ml of basal medium produced 38, 51, and 49% as much stimulation, respectively, as was obtained with 20% rumen fluid added to basal medium. The effects of rumen fluid in combination with mucin were not additive. Similarly, fresh bovine saliva in concentrations of 1 and 2% of the basal medium gave stimulation equivalent to 50 and 37% of that obtained with 20% rumen fluid added to basal medium. Of the constituents reported to be in bovine saliva by Reid and Huffman (10), only mucin caused growth stimulation. Mucin has been used for many years to enhance the virulence of certain pathogens in laboratory cultures. In addition to stimulation of rumen cultures by microbial products, the observed stimulation of *B. fibrisolvens* by mucin may further explain the findings of several authors that stimulatory activity is absent from dried grass, malt sprouts, and other materials in the ruminant diet (2, 9, 11).

That part of the stimulatory activity of rumen fluid which could not be replaced by mucin was not replaced by any single one of 18 amino acids, nine vitamins, eight sugars, or seven other metabolites commonly used to stimulate cultures.

Dialyzed, autoclaved rumen fluid was chromatographed on Whatman 3-MM paper. The chromatograms were developed with methyl-ethyl ketone, isopropyl alcohol, ammonium hydroxide, and water in the ratio of 3:5:1:1 in a descending system. Stimulatory activity remained in the area between Rf values of 0.028 and 0.086. Elution into basal medium of material in the area bounded by Rf values of 0.114 and 0.318 caused complete inhibition of growth. The presence of this inhibitory factor in the rumen fluid was substantiated by the absence of inhibition by all eluates from control chromatograms which were developed without rumen fluid. The nature of the growth inhibitor was not determined.

G. R. GORDON [1]
W. E. C. MOORE
Virginia Agricultural Experiment Station
Blacksburg, Virginia

REFERENCES

(1) ALLISON, M. J., BRYANT, M. P., AND DOETSCH, R. N. A Volatile Fatty Acid Growth Factor for Cellulolytic Cocci of the Bovine Rumen. Science, 128: 474. 1958.
(2) BRYANT, M. P. The Isolation and Characterization of a Spirochaete from the Bovine Rumen. J. Bacteriol, 64: 325. 1952.
(3) BRYANT, M. P., AND BURKEY, L. A. The Bacterial Flora in the Rumen of Heifers Fed a Ration of Alfalfa Silage. Bureau Dairy Ind., Inf. Bull. 151. 1953.
(4) BRYANT, M. P., AND DOETSCH, R. N. A Study of Actively Cellulolytic Rod-Shaped Bacteria of the Bovine Rumen. J. Dairy Sci., 37: 1176. 1954.
(5) BRYANT, M. P., AND DOETSCH, R. N. Factors Necessary for the Growth of *Bacteroides succinogenes* in the Volatile Acid Fraction of Rumen Fluid. J. Dairy Sci., 38: 340. 1955.
(6) BRYANT, M. P., AND SMALL, N. TheAnaerobic, Monotrichous, Butyric Acid–Producing, Curved, Rod-Shaped Bacteria of the Rumen. J. Bacteriol., 72: 16. 1957.
(7) DEHORITY, B. A., BENTLEY, O. G., JOHNSON, R. R., AND MOXON, A. L. Isolation and Identification of Compounds from Autolyzed Yeast Alfalfa Meal, and Casein Hydrolysate with Cellulolytic Factor Activity for Rumen Microorganisms *in Vitro*. J. Animal Sci., 16: 502. 1957.
(8) GILL, J. W., AND KING, K. W. Nutritional Characteristics of a *Butyrivibrio*. J. Bacteriol., 75: 666. 1958.
(9) HUNGATE, R. E. The Anaerobic Mesophilic Cellulolytic Bacteria. Bact. Rev., 14: 1. 1950.
(10) REID, J. T., AND HUFFMAN, C. F. Some Physiological and Chemical Properties of Bovine Saliva Which May Affect Rumen Digestion and Synthesis. J. Dairy Sci., 32: 123. 1949.
(11) RUF, E. W., HALE, W. H., AND BURROUGHS, W. Observations Upon an Unidentified Factor in Feedstuffs Stimulatory to Cellulose Digestion in the Rumen and Improved Liveweight Gains in Lambs. J. Animal Sci., 12: 731. 1953.

[1] Present address: Bacteriology Department, Research Center, Christ Hospital, Cincinnati, Ohio.

PALMITALDEHYDROGENIC SUBSTANCE IN BOVINE SPERM LIPIDS [1, 2]

While investigating the fatty acid composition of bovine sperm lipids, we noted the presence of a peak on gas chromatograms that could not be identified as any known fatty acid methyl ester. In the analysis of six samples of sperm lipids this peak was alwayys present and, as indicated by its peak area, represented a quantitatively significant component.

Methyl esters of fatty acids in sperm lipids were prepared for GC analysis as follows: Pooled ejaculates of freshly collected bovine semen were added dropwise to an equal volume of ethanol, mixed, and one volume each of petroleum ether and ethyl ether added. After extraction, the extract was evaporated to dryness and saponified in methanolic potassium hydroxide. The saponified material was extracted with ethyl ether to remove unsaponifiable matter, the ethyl ether discarded, and the residue acidified and extracted with petroleum ether. Fatty acids were recovered by evaporating the petroleum ether and methyl esters prepared by refluxing for 1 hr. in methanol

[1] Authorized for publication as Paper No. 2568 in the Journal Series of the Pennsylvania Agricultural Experiment Station on June 13, 1961.
[2] Supported in part by the U. S. Public Health Service (H3632).

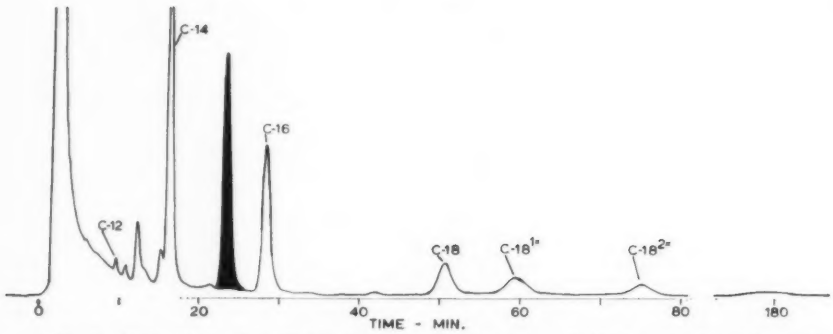


FIG. 1. Separation of methyl esters from bovine sperm lipids by gas chromatography. Operating conditions: 9-ft. by ¼-in. coiled column packed with 17 wt. % ethyleneglycol succinate polyester on 80-100 mesh celite; column temperature, 182° C.; argon pressure, 20 p.s.i.

containing 0.5% sulfuric acid. After esterification the reaction mixture was cooled, diluted with an equal volume of water, saturated with sodium chloride, and the esters extracted into petroleum ether. Methyl esters were thus prepared from whole semen, washed sperm, and seminal plasma. The preparations from whole semen and washed sperm yielded essentially identical gas chromatograms, whereas the seminal plasma appeared devoid of lipids.

The methyl ester samples were analyzed with Barber-Coleman Models 10 and 20 gas chromatography instruments, employing EGS and Apiezon L columns for identification of the esters by procedures previously described (4). A representative chromatogram for methyl esters of bovine sperm lipid fatty acids is shown (Figure 1). The darkened peak, falling between those for the C_{14} and C_{16} components, is due to the unknown in question. The saturated C_{14}, C_{16}, and C_{18} acids accounted for over 80% of the fatty acids in the sperm lipid samples analyzed.

A clue to the identity of the unknown component was obtained when treatment of the methyl esters from sperm lipids with dinitrophenylhydrazine was observed to eliminate the unidentified peak from the gas chromatogram. After treatment with this reagent, detection of dinitrophenylhydrazones in the sample was attempted with the paper chromatographic method of Klein and de Jong (3). A prominent spot corresponding in Rf value to palmitaldehyde dinitrophenylhydrazone was observed. Using the identification procedure for fatty aldehydes of Gray (2), involving the use of both polar and nonpolar gas chromatography columns, it was established that the unknown on the gas chromatogram of sperm lipid methyl esters corresponds precisely in retention time to authentic palmitaldehyde dimethyl acetal. In like manner, the small peak at 42 min. on the chromatogram (Figure 1) was identified as stearaldehyde dimethylacetal.

Gray (1) has shown that the plasmalogen fraction of the lipids from ram semen is rich in bound palmitaldehyde and contains lesser amounts of other bound aldehydes. From the present results, it appears that this is also true of lipids from bovine semen and, more particularly, from the spermatozoa.

Cleavage of the aldehyde (vinyl ether) moiety from a plasmalogen yields a lysophosphatide. These types of compounds are effective in bringing about structural changes at the cellular level; for example, lysis of red blood cells (6) and disintegration of mitochondria (5). Since conception involves penetration of egg by sperm and dissolution of sperm within the egg, plasmalogens may play an important role in the process. On the other hand, if the spermatozoa owes its structural integrity to plasmalogens, their hydrolysis by an unfavorable (acidic) uterine environment is conceivable as the basis of a female sterility problem.

STUART PATTON
ROBERT J. FLUSE
Department of Dairy Science
Pennsylvania State University
University Park

REFERENCES

(1) GRAY, G. M. The Phospholipids of Ox Spleen with Special Reference to the Fatty Acid and Fatty Aldehyde Composition of the Lecithin and Kephalin Fractions. Biochem. J., 77: 82. 1960.
(2) GRAY, G. M. Separation of the Long-Chain Fatty Aldehydes by Gas-Liquid Chromatography. J. Chromatography, 4: 52. 1960.
(3) KLEIN, F., AND DE JONG, K. Paper Chromatography of 2,4-Dinitrophenylhydrazones of Aliphatic Carbonyl Compounds. Rec. trav. chim., 75: 1285. 1956.
(4) PATTON, S., MCCARTHY, R. D., EVANS, LAURA, AND LYNN, T. R. Structure and Synthesis of Milk Fat. I. Gas Chromatographic Analysis. J. Dairy Sci., 43: 1187. 1960.
(5) ROSSITER, R. J., AND STRICKLAND, K. P. In Lipide Metabolism, K. Bloch (ed.). p. 97. John Wiley and Sons, New York. 1960.
(6) SUMNER, J. B., AND SOMERS, G. F. Chemistry and Methods of Enzymes. 3rd ed. p. 77. Academic Press, New York. 1953.

OUR INDUSTRY TODAY

PESTICIDE RESIDUES AND THE DAIRY INDUSTRY [1]

H. E. O. Heineman and C. B. Miller
Pet Milk Research Center, Greenville, Illinois

There has been increasing awareness and concern on the part of food and health officials and of the dairy industry about the presence in milk and milk products of pesticide residues. The problem is not peculiar to dairy products. All raw agricultural commodities have been undergoing a similar trend which parallels the tremendous increase during the past twenty-five years in the use of pesticides in this country. Agriculture, as we know it today, could not exist without them. More than 200 basic chemicals are now being produced and offered for sale and use on our farms. The upward trend is certain to continue as well as it should. Consumers have come to expect products with quality and sanitation standards never dreamed of in past generations. Pesticides offer the only economical means of maintaining not only this quality but also the adequacy and sufficiency of agricultural production.

IMPACT OF THE MILLER BILL

Milk production and manufacture became directly involved in the pesticide residue problem with the passage in 1954 of the Miller Bill, now known as the Pesticide Amendment to the Food, Drug, and Cosmetic Act, which provides Food and Drug Administration with authority to establish and enforce tolerances for pesticide chemicals in raw agricultural products. Since that time, more than 2,000 such tolerances or exemptions from the requirements of tolerances have been established for a wide variety of pesticide chemicals in basic agricultural products.

There is an interesting aspect to this amendment which may not be generally realized, which is that producers, processors, or manufacturers of agricultural products are not permitted to petition for tolerances for pesticide chemicals, for example, in milk or milk products. The law reserves this right to the manufacturers of the chemicals, which must already have been registered with the U. S. Department of Agriculture under the Federal Insecticide, Fungicide, and Rodenticide Act. However, the Food and Drug Administration at its discretion can establish tolerances in the absence of a petition and has done so on frequent occasions.

POLICY OF REGULATORY OFFICIALS

It has been the traditional policy of food and health officials, including the Federal Food and Drug Administration, to oppose the addition to milk in any amount of poisonous or deleterious substances. In the absence, therefore, of any established tolerances for milk and dairy products, Food and Drug Administration has followed an enforcement policy based on zero, i.e., that any residues found in milk are violative of the Pesticide Amendment. In consideration of these policies, manufacturers of agricultural chemicals have usually not included milk among the agricultural products for which they have applied for tolerances for their respective pesticides.

There has been one notable exception: The manufacturer of Methoxychlor did include in his petition a request for a tolerance in milk for this particular chemical. Scientific evidence of the type generally accepted as adequate for establishing safety of chemicals in foods was submitted. At the request of the Food and Drug Administration, a special committee was nominated by the National Research Council to consider this petition. After due consideration, the committee recommended against the requested tolerance for Methoxychlor in milk, even at the requested low level of 0.25 p.p.m., and the petition was denied.

The significant factor here is not the denial of the petition but, rather, is contained in the reasons given by the committee for its recommendation:

> ". . . that because of the unique position of milk in the diet of infants and normal and ill adults, a greater margin of safety must be established in a proposal for a pesticide tolerance petition than was demonstrated (in the petition for Methoxychlor) and, in fact, a greater margin of safety than would be the case for any other food item in the human diet."

Going beyond this statement, the committee recommended that FDA remove milk from its list of raw agricultural commodities under the Pesticide Amendment. This indicated the committee's belief that there should be no provision in law for the establishment of any tolerance for pesticide chemical residues in milk.

UNIQUE POSITION OF MILK

Most technical men would agree with part of the committee's conclusion, i.e., as to the unique position of milk in the diets of special segments of the population—that milk enters the diet more frequently and in greater amounts than other foods for which tolerances have been granted—that, therefore, a greater margin of safety should be allowed in regulating pesticide residues in milk as compared with most

[1] Presented at the 56th Annual Meeting of the American Dairy Science Association, University of Wisconsin, Madison, 1961.

other foods. On the other hand, it is difficult to discern any scientific basis for the committee's recommendation that there be no provision in law for tolerances for these residues in milk. The reasons are simple. Pharmacologists agree that for every chemical registered for agricultural use, there is a safe level which can be scientifically determined. Moreover, even under the best agricultural practices, it is impossible to produce milk in today's environment which is completely free from pesticide residues. With present methodology, it may not always be possible to detect the presence of such residues, but they are there, nonetheless. A vast amount of data establishing this is now available.

PRACTICAL CONSIDERATIONS

In the intervening years, since the passage of the Pesticide Amendment, the dairy industry has taken a somewhat detached view of the pesticide residue problem. It has been common knowledge that residues are present in milk. But viewed in the light of the tolerances established for many other agricultural products, for example, 7 to 14 p.p.m. DDT in or on fruits, vegetables, the fat of meat, etc., residues in milk, according to the available data, are well within or below these levels. There are other factors which in recent years held back action programs to monitor or control the residue problem. There is no analytical method which can be used at the plant level for testing incoming milk for pesticide residue content. Even for the analysis of finished milk products, methodology is time-consuming and expensive and, up until very recently, the only available methods determined merely total organic chlorine which was, in fact, a measure of DDT and its homologues and provided no identification or quantitative measure of other compounds.

Moreover, there had not been general awareness in the dairy industry of the behavior of the chlorinated hydrocarbon group of chemicals, which have the unfortunate characteristic of being transmitted through the biological system of the cow into milk. They can enter the biological system through the skin, by inhalation, or by ingestion. They are stable in milk, unaffected by heat processing. In varying degrees, they are stored in the fat of the animal and above certain thresholds translocate into milk over prolonged periods of time. There are data which indicate, for example, that DDT continues to appear in the milk of a dairy cow for months after the source is removed, the time depending upon the rate of feeding or upon the rate of depletion of stores of body fat.

In the face of some of these obstacles, little progress was made in controlling pesticide residues in milk. Nevertheless, however mitigating these factors may be, the dairy industry must face up to and acknowledge negligence in its approach to the total problem.

The situation was changed radically when, in late 1959 and early 1960, FDA seized shipments of dairy products moving in interstate commerce on the basis of pesticide residue content. The amounts in the milk were small—approximately 0.2 p.p.m. on the fluid milk basis or 4 to 5 p.p.m. in the fat as determined by the Schecter-Haller Method. This is the official method, with a reliability probably not below about 2.5 p.p.m. in milk fat. The method is probably adequate for enforcement purposes but not for a technical study of the incidence, types, levels, or sources of pesticide residues in milk.

AVAILABLE METHODS

Fortunately, a good screening method is now available, developed by Paul Mills of FDA and published in the *Journal of the Association of Official Agricultural Chemists* in November, 1959. It employs paper chromatography and can be used for the identification and semiquantitative estimation of chlorinated hydrocarbons. It will detect DDT, DDE, TDE, Heptachlor Epoxide, Lindane, Benzene Hexachloride, Methoxychlor, Chlordane, Perthane, Heptachlor, and Toxaphene. The Mills Method has been given first action by the A.O.A.C. for the first six pesticide residues listed above in dairy products. Although it was adopted as a method for identification only, it does have semiquantitative analytical value. In our laboratory, the method has sensitivity down to roughly 0.3 p.p.m., in the fat of dairy products.

In the wake of events described above, the dairy industry under the auspices of its Dairy Industry Committee appointed a Technical Advisory Committee to appraise the pesticide problem and to develop an action program. This committee is made up of 12 technical men representing leading corporate and cooperative manufacturers of dairy products in the United States. Here are some of the questions to which the committee undertook to find answers: What pesticide residues occur in milk? What is the incidence of these residues? What are the levels? What, if any, are the seasonal influences and geographical variations? What are the sources of these chemicals? Are the levels in milk such as to constitute a public health hazard? What is the best methodology for measuring residues? How sensitive are the methods? Is it possible or practical to produce residue-free milk? On what basis could the residue problem be resolved which would be mutually satisfactory to food and health officials and to the dairy industry? Such a basis would need, obviously, to integrate the objectives of both groups in terms of the residue levels in milk which (1) are biologically insignificant and (2) are the lowest which can be achieved under good agricultural practices.

COMMITTEE ORGANIZATION

The Technical Advisory Committee was organized into six subgroups:

(a) Methodology and collection of analytical data.
(b) Sources of pesticides in milk. It has been FDA's contention that the chief source is poor dairy-barn practices, i.e., the misuse of pesticides by the dairy farmer. It was necessary to find out if this were true or if, on the other hand, feeds and forage might be the major contributor.
(c) Toxicology.
(d) Survey of the scientific literature.
(e) Liaison with the feed industry.
(f) The Dairy Industry Committee itself acting to conduct a strong educational program for dairy farmers.

The committee held its first meeting in May, 1960, and thus has now completed a full year of work. It has been an inspiring experience to the writer to chairman this group of technical leaders in the industry, who have given so unstintingly of their time to develop and interpret information and to implement this research and educational program.

Twelve laboratories have collaborated in the collection of data. More than 20,000 analyses have been submitted, representing all types of dairy products and all geographic regions in the United States. A full year's cycle has been completed, which delineates seasonal variations. Sources of pesticide contamination have been studied. Data on balance studies in dairy cows are available which throw light on the ratio of intake of pesticides in feed to exeretion in milk. A review of toxicologic data on the chlorinated hydrocarbons has been made and a report prepared on this important aspect. The survey of literature now being printed includes some 800 abstracts of pertinent technical articles.

DATA COLLECTED BY PET RESEARCH CENTER

A progress report is being prepared for presentation to FDA within hte next few weeks. Therefore, I shall not pre-empt that report by presenting the committee's findings here. In lieu thereof, I should like to present a study from our own laboratory, which I believe reveals with some clarity the present status of the pesticide residue problem with respect to incidence of residues, levels, and sources of contamination.

This particular case study, although representing only a portion of the total data collected by the committee, nevertheless has certain advantages from the standpoint of reliable interpretation. It includes approximately 4,000 analyses of dairy products. All of the analyses were conducted in the same laboratory, by the same personnel, and by the same method. The Mills Paper Chromatography Method was strictly followed. The sampling is consistent, since the same plant locations were represented. The study covers a complete 12-month cycle. The local dairy farmers were subjected to the same educational program at all sampling locations.

In our laboratory, about 53 separate manipulations are necessary when the method is applied, for example, to evaporated milk. Quantities as low as 0.2 μg. can be identified, equivalent to 0.3 p.p.m. on the fat basis. Recovery

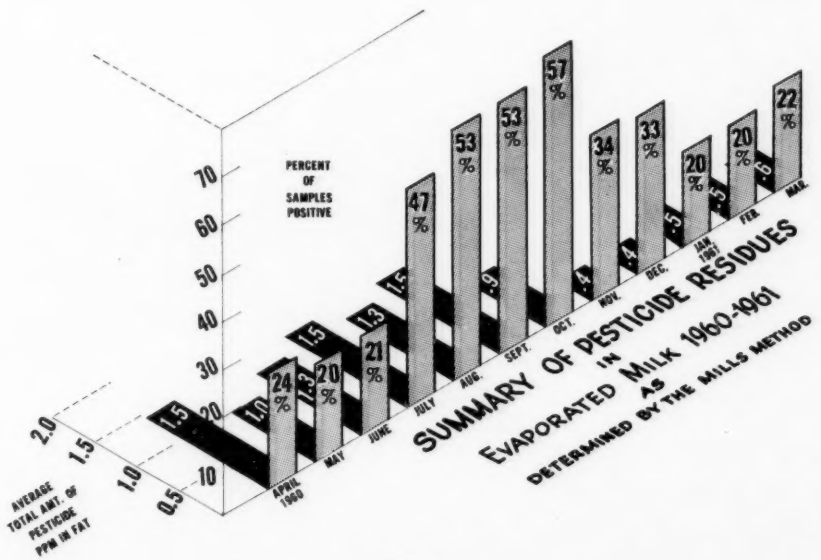


FIG. 1

studies indicate that in our laboratory the method recovers between 60 and 80% of added pesticides. The data reported have been corrected, assuming an average recovery of 70%. Studies have been conducted to evaluate the minimum amount detectable. As might be expected, this value has changed over the year of study as personnel became more efficient in the techniques. This shifting of the lower limit of detectability causes some slight difficulty in interpretation of the year's data, but this will be explained as we view the following slides.

In all instances where levels of pesticides are shown, the level is the estimated total amount of residue in the fat. This is obtained by adding together all the residues found in each sample. Therefore, the values represent the aggregate of the chlorinated hydrocarbons in the particular milk product being analyzed.

RESULTS AND DISCUSSION

Figure 1 shows the level and incidence of pesticide residues on almost a country-wide basis month by month. The seasonal characteristics of the residue problem are shown in the marked increase in incidence from July through October. The average levels remained relatively high throughout the summer and fell off sharply in the fall. Levels found in March, 1961, are significantly lower than in the corresponding period of 1960.

Figures 2, 3, and 4 depict the same type of information as in Figure 1, but are grouped according to geographic sections of the United States. Figure 2 is the northern area—Wisconsin, Ohio, Illinois, Kansas, Missouri. Here both levels and incidence rose slowly throughout the summer months to a peak in October, then fell off sharply for the remainder of the months reported here. Figure 3 shows a comparable situation for the southern area—Virginia, Maryland, Tennessee, Kentucky, Mississippi, and Arkansas. It may be noted that a plateau was reached in July and maintained through October. Levels were relatively high during this period.

Figure 4 reveals a serious problem area in the United States, i.e., the Mountain and Pacific regions—Idaho, Utah, California. Incidence was significantly higher than in other geographic areas. The peak of incidence came somewhat earlier here. Levels after the month of April show a rather characteristic rise and fall, with the peak levels appearing slightly before the peak incidence.

Although one must use caution in drawing too narrow conclusions from these data, there is strong indication in all areas that incidence of pesticide residues in milk follows the pattern of the major growing seasons. It would follow that this would be the time of greatest activity in the spraying or dusting of agricultural crops.

It would appear that little progress has been made in reducing the incidence of contamination of milk with residues. However, it should be borne in mind that in this particular study, during the last six months, levels were being detected that might have been missed in April and May of the previous year, 1960.

Importantly, levels of residues found have definitely decreased when compared to results a year ago. A factor which may have some bearing upon the apparent lack of a lowering

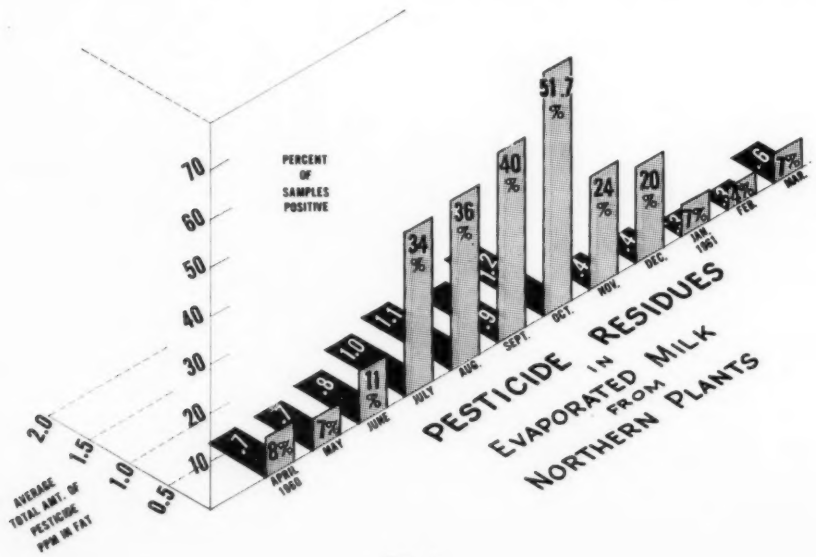


FIG. 2

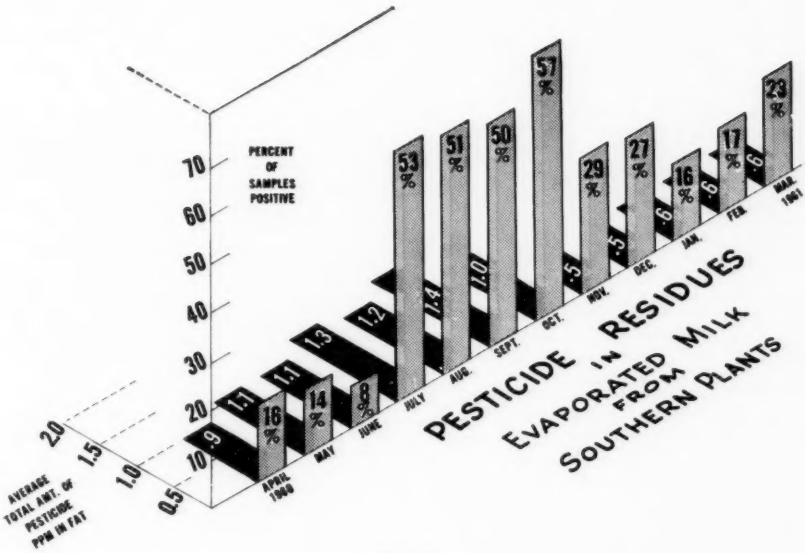


FIG. 3

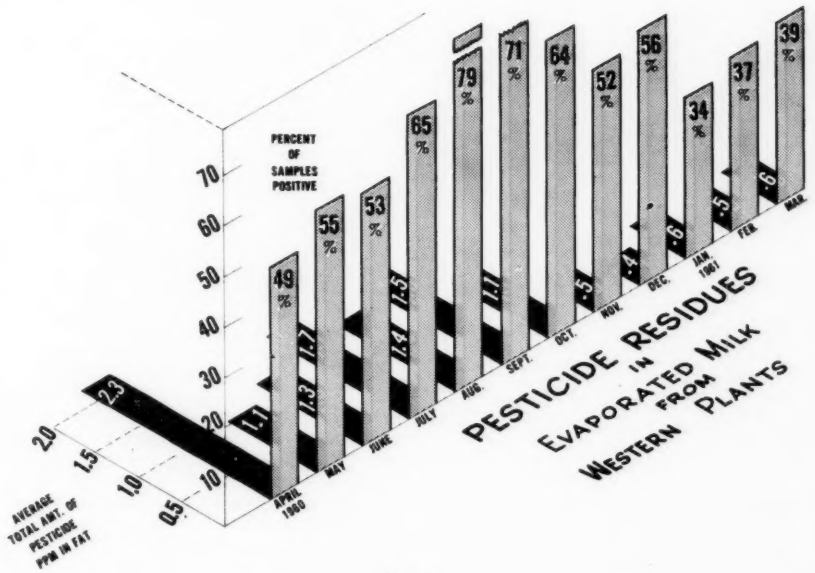


FIG. 4

of incidence is the extreme persistence of these residues in the biological system of the cow. Studies reported in the literature show retention times from 120 days up to a year in some instances. Our own study has shown a retention of DDT up to ten months. Under such circumstances, rapid elimination of detectable residues would not occur.

Figure 5 shows the specific residues found in these 4,000 samples. Approximately 90% of all positive samples contained DDT; 43% contained DDE, a homologue of DDT. This

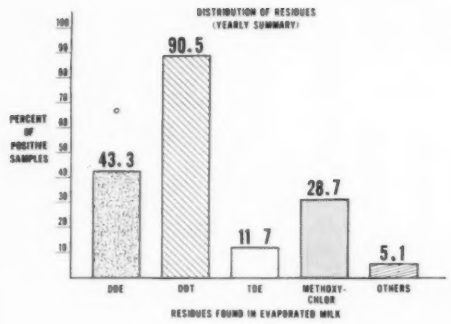


FIG. 5

is probably the result of the breakdown of DDT, either by weathering or metabolic systems. Twelve per cent of the samples contained TDE or DDD, which may be the result of direct application of these chemicals to crops or can also represent breakdown of DDT. The 29% of positive samples showing Methoxychlor will be discussed later. Only 5% of the positive samples contained residues other than the four mentioned above. A few of these were Lindane, some BHC, but the majority represented an unidentified residue appearing in cotton-growing areas. Efforts to identify this compound have failed. We know only that it is not one of the more commonly used chlorinated hydrocarbons. It can be postulated that it is a metabolite of a pesticide or herbicide used in the production of cotton.

Figure 6 shows the seasonal use of Methoxychlor. The first peak in July very likely coincides with the maximum fly-population season. The second peak in October is more difficult to explain. It may be the result of usage to protect cattle against other pests, such as face flies or gnats. Since Methoxychlor has a very short biological retention time, the sharp peaks are not unexpected. Of all the data gathered, those concerning Methoxychlor are the only indication of misuse of a pesticide on the dairy farm. Data in the literature, as well as our own experience indicate, however, that Methoxychlor can be used in such a manner that it will not appear in the milk. Continuing effort in educating the farmer in the proper use of Methoxychlor is needed.

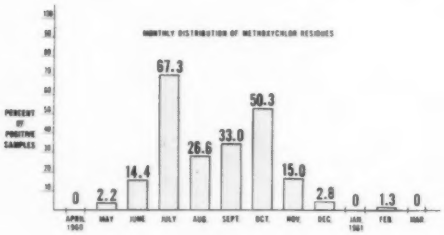


FIG. 6

Figure 7 clarifies the situation with respect to DDT residues. At least 90% of all positive samples contained detectable amounts, reflecting the widespread use in agriculture of DDT and its long biological retention. It is interesting, however, that extensive surveys indicate that the use of DDT on dairy animals and in dairy barns is negligible. These are highly significant findings, pointing directly to feeds and forage as being substantial contributors of DDT residues in milk.

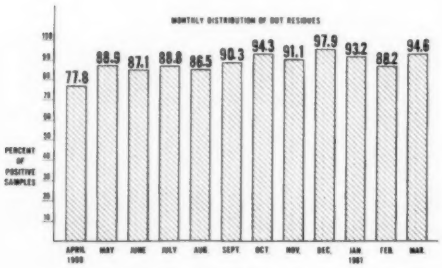


FIG. 7

Figure 8 shows the range of levels of total residues in the fat of dairy products. The rather sudden change appearing in November needs interpretation. It was during October that this laboratory recognized its ability to detect lower levels of contamination as techniques improved. At this point, we started carrying smaller standard amounts on papers than previously and were thus able to grade the papers down further than in the earlier work. When these smaller amounts were recognized, this obviously resulted in samples whose aggregate residue content was evaluated at lower levels. Although the accuracy of the chart is thus slightly impaired, as a result of this changing technique in the quantitative evaluation of the paper chromatograms, nevertheless the broader indication is clear, i.e., that

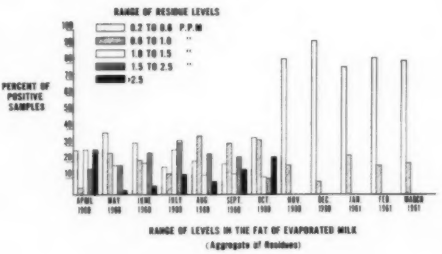


FIG. 8

during the period April, 1960, through March, 1961, aggregate residue content has been significantly reduced in all areas included in this study.

SUMMARY

Study of the data derived from this work indicates that progress in controlling the contamination of milk by pesticides has been made. Residue levels have been reduced in the year reported here. The fact that incidence of residues has not been materially reduced indicates the complexity of the problem. Biological retention of chlorinated hydrocarbons is an established fact and undoubtedly is contributing to this continued appearance of low-level residues in the milk supply. Personal contact by field men with producers supplying milk to the various plants sampled in this program reveals that actual in-barn and on-animal usage of chlorinated hydrocarbons, except for Methoxychlor, has virtually been eliminated. This leaves the relatively unknown area of the conditions of pastures and feeds as a route of continuing contamination. Many of these conditions are beyond the control of the dairy farmer. There is indication that more stringent control of the use of Methoxychlor should be exercised by the dairy farmer.

The results of the study point to the unrealistic nature of an absolute zero tolerance for pesticides on an agricultural product that is produced in an environment shared with other agricultural products upon which pesticide usage is permitted and necessary.

The contention of some food and health officials that, by and large, milk can be produced free of residue is not borne out. Moreover, the data indicate that probably no dairying area produces milk completely free of residue. When a residue is not found, it can be said only that the sample does not contain a sufficient amount to be detected. It is probable that if our methodology were consistently sensitive to lower levels, the incidence of contamination would be found higher than at present. There is no zero for the analytical chemist; there is only an amount below which he can not detect a substance.

It becomes increasingly clear that a strong educational program directed to dairy farmers on the proper use of pesticide chemicals, and on selection of feeds and forage on which pesticide application is controlled, in a comparatively brief time, i.e., twelve months, can produce significant reduction in levels of residues in milk. However, we do not have evidence that incidence of contamination is materially reduced.

It is apparent from these studies that under such a program, pesticide residues in milk and milk products can be controlled at levels sufficiently low as to constitute no hazard to public health.

The data show that feeds and forage are suspect as major contributors of pesticide contamination in milk and that action programs in the feed and related agricultural industries are probably necessary to bring about further improvements.

Above all, these studies indicate the necessity for a persistent, industry-wide action program to monitor and control pesticide residues from any source which may contaminate dairy products, so as to preserve the safety and reputation of milk and its products, as major components of the diets of all segments of the population.

ASSOCIATION AFFAIRS

PUBLICATION PROBLEMS OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

E. L. Jack
Department of Food Science and Technology, University of California, Davis

As President of the American Dairy Science Association, it is my privilege as well as my duty to discuss with the membership from time to time problems of the Association and other matters of common interest.

One area of immediate and substantial concern is that of publications. There are actually two problems here, although they are interrelated in some respects. One is to determine just what should be the scope of Association publications; can the responsibilities be met with the present journals or should substantial changes be made? The other problem is how to meet the cost of publishing the increasing number of scientific papers.

E. L. Jack

The problem of the scope of publications revolves around the need to serve both the scientists and those members primarily interested in practical applications. We have always had this problem, but it did not become acute until dairy science progressed to the point where much of the research is basic in nature and the applications are not readily apparent. Additions of new sections to the Journal of Dairy Science have been made from time to time, attempting to serve more satisfactorily those with diverse interests. The latest one which has culminated in the quarterly, The A.D.S.A. Review, has brought into sharp focus the need to examine the policy critically and to evaluate plans for meeting the Association's responsibility to the entire membership.

The view has developed over a period of years that the needs of the membership can best be served by two different publications, as is done by many similar scientific societies: one devoted strictly to scientific research and the other technical, or semitechnical, carrying news items, applications of research, reports of applied research, and other matters of general interest.

In considering what is most desirable, one must always take into account what is possible or feasible. Therefore, to come to grips with this problem and determine what can be done, the Executive Board at the Madison meeting directed the Journal Management Committee, under whose jurisdiction this matter falls, to study the feasibility of the Association publishing two journals and, if feasible, to outline plans for implementing and financing the venture. If not feasible, alternative possibilities must be considered. This Committee is to report at the next meeting of the Executive Board, after which some concrete proposals can be reported to the membership.

The problem of publication costs is one which concerns vitally every scientific society. It is critical to A.D.S.A. at the present time, because the cost of publishing the Journal will exceed in 1961 that of the previous year by about $1,000 per month. This will result in a substantial deficit for the Association, and steps have been taken by the Executive Board to try to balance the budget for 1962.

However, the problem will be continuously recurring in the foreseeable future, unless some radical changes are made. This increase of cost results largely from an increased research productivity. The number of journal pages for the first four months of 1961 averaged about 40 more per issue than for 1960. And this increase in the quantity of research will continue. During the last few years, relatively large sums of money from federal grants and elsewhere have become available for all kinds of scientific research. In the field of dairy science the impact of this expanded activity is just beginning to show up in manuscript output. Even greater expansion is sure to come. How can this be dealt with?

Most scientific societies are concerned over this problem and are actively examining it. Dr. Louis P. Hammett of Columbia University devoted his Priestley Medal address at the March meeting of the American Chemical Society at St. Louis to this problem. To date, however, so far as I know, the problem of how to deal with this increasingly great mass of scientific manuscripts is still in the discussion stage; no one has made any remedial changes in their procedures. Certainly, it is highly desirable to continue to encourage scientific research and its subsequent publication. And just as certainly, there is a limit to which we can go in passing on to the reader the costs of publishing such research. Many people think that we have about reached that limit now; increased subscription rates usually lead to a decrease in the number of subscribers.

One of the suggested remedies, and one which I think is sound in principle, is to require the producer of the research to assist financially in its publication. With the large amount of

money now available for research, only a small percentage would be required for publication costs. The Institute of Physics has long made a charge for publishing in its journal. Such a procedure, it seems to me, has three desirable characteristics: first, it should encourage brevity and thus more readable papers; secondly, it should discourage the submission of papers of questionable quality; and thirdly, it would make the cost of reporting the research an integral part of the entire project. However, its adoption would require concerted action by a number of societies.

There are other ways in which these mounting publication costs may be met. One, of course, is to limit arbitrarily the size of the JOURNAL. To date your Board has not discussed seriously anything other than routine adjustments. Nevertheless, the problem is with us and will reach crisis proportions before too long. The expressions and suggestions of the membership will be valuable guides to any action the Board may take. Send them to any member of the Board, along with any other suggestions you may have. They will be welcome.

CAN THE JOURNAL OF DAIRY SCIENCE BE USEFUL TO LATIN AMERICAN COUNTRIES?

EARL WEAVER
Dairy Department, Michigan State University, East Lansing

Men from the United States, who have some knowledge of dairy conditions in Latin American countries, entertain no doubts that our JOURNAL OF DAIRY SCIENCE could be useful in those countries. My full realization of this value was reached through the opportunity I had in serving a 4-yr. period (1955 to 1959) as a staff member from Michigan State University on the Technical Aid mission to the College of Agriculture at Medellin, Colombia.

During my assignment to Colombia, special efforts were exerted to visit other countries, too. Visits were made to Ecuador, Peru, Chili, Argentina, Uruguay, Brazil, and Venezuela. From nearly all contacts and observations that were made, it was apparent that the JOURNAL could be of extreme value to dairy teachers, students, research workers, and other dairy leaders.

There are many agencies in the United States that are dedicated to Technical Assistance for so-called underdeveloped countries. These agencies have accomplished a great deal of good. From my observations, I would conclude the activities in Technical Assistance in past years can be rated higher than some other activities under the over-all program of Foreign Aid. The work in Technical Assistance has been less costly to the United States, has afforded more benefit to the people it is supposed to serve, and has provoked fewer boomerangs against our country.

It would seem that some of these agencies offering Technical Assistance might respond eagerly to a proposal from our Association to expand their efforts through such a medium as the JOURNAL OF DAIRY SCIENCE. The JOURNAL could wield an influence in advancing dairy research efforts and education in the countries where a dire need of the people is the improvement in the science and technology that are essential for the abundant production of suitable foods.

However, we members of the American Dairy Science Association must be realistic as we contemplate the possibilities for the JOURNAL to be made most serviceable in Latin America. In terms of the peso or of any other monetary unit in Central or South America, the relative cost of a membership or subscription is a deterrent. Currently, there are only some 25 or 30 association members in the whole of Latin America. This averages fewer than two members per country. In most instances the research workers, teachers, and other interested dairymen in Latin America would use the JOURNAL effectively if it were available to them at a cost commensurate with their salaries. Currency exchange rates affect not only the prospective members but also JOURNAL subscriptions by individuals, libraries, and commercial dairy concerns.

Maybe one or more of the agencies that are engaged in Technical Assistance could help solve this cost problem through a partial subsidy for memberships and subscriptions. Maybe A.D.S.A. itself could modify its rates for foreign members and subscribers.

It would not be advisable to relieve prospective members or subscribers in Latin America of all the costs; they should be expected to bear some themselves.

As the usefulness of the JOURNAL in the countries of Central and South America may receive consideration, a second problem, that of the language, must be recognized. It is not too serious. In all these countries dairy teachers and research workers can speak English quite fluently; they handle written English with equal ease—or even better. On the other hand, some of the good teachers and research workers,

as well as most university students, are not proficient in English. Even to read it they have to exert considerable painstaking effort—often with the tedious use of a dictionary.

To meet the language barrier, I could scarcely conceive of the feasibility of a Spanish translation of the entire JOURNAL. However, we have the newly established A.D.S.A. Review. The costs in providing a Spanish edition of the Review would not be prohibitive.

Again it would seem that some agency now engaged in Technical Assistance might be interested to underwrite the costs of such an edition.

My teaching work in Colombia led to my realization of the lack of suitable texts and resource material available in Spanish for class use. A Spanish edition of the A.D.S.A. Review would be of inestimable value to dairy teachers; it would make their teaching easier and more effective; it would make the students' work far more satisfying.

CALL FOR PAPERS FOR THE 1962 ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

M. E. SENGER, Chairman, Program Committee, A.D.S.A.
Department of Animal Industry, North Carolina State College, Raleigh

The 57th Annual Meeting of the American Dairy Science Association will be held June 18-21 at the University of Maryland, College Park. Members who wish to present papers must submit titles and abstracts not later than March 1. This deadline must be met to permit publication of titles with the complete program in the April, and abstracts in the June, JOURNAL.

All members of the Association, including graduate student affiliates, are entitled to present papers. Participation by members of the industry and by senior members of the Association is especially encouraged. The Program Committee favors the general policy that an individual present only one paper and that his name appear as author on no more than two. This Committee, together with the Association membership, wishes to stimulate vigorous, sound research and realizes that these restrictions may penalize some members engaged in full-time research. Therefore, the Committee has liberalized this policy so that a member may exceed these limits, but only if he or his department rates the abstracts in order of preference for oral presentation.

Papers submitted for the annual meeting should be confined to research that has not been reported. Abstracts of papers accepted for publication by a scientific journal before the annual meeting are not eligible, as this constitutes duplication. If the total number of papers submitted by the membership is too great to include in the program, the Committee will assign some papers to be read by title only. In this event, consideration will be given to quality of research to be reported, number of abstracts per author and department, and importance of the research. Abstracts arriving late will be rejected.

Attention is called to the Dairy Manufacturing Extension Section program and members are urged to participate. This is a subsection of the Manufacturing Section.

Mimeographed copies of pertinent data are desirable for distribution when the paper is presented. At least 250 copies should be made available. This can be supplemented by slides for projection on screens, provided the author can adhere to the assigned time of 12 to 14 minutes for presentation of each paper.

PREPARING ABSTRACTS

The Program Committee encourages continued improvement in quality of papers and in oral presentation. Careful design of experiments and proper interpretation of results are necessary. Strict compliance with the instructions for preparation of abstracts will simplify the task of the Program Committee, and will improve the program. *Careful editing of abstracts before submission is essential.* Each year a number of abstracts must be returned due to incomplete details. Please follow instructions for preparing abstracts carefully.

1. All abstracts must be submitted on regular 8½- by 11-inch paper.
2. Abstracts must not exceed 200 words by actual count. Those exceeding 200 words will be returned to the author for revision.
3. An original (on bond paper) and three copies of each abstract should be typed double-spaced. The original and one copy should be mailed to the Chairman, another copy to the Vice-Chairman, and the fourth to the Secretary of the Section where the paper will be presented. The original copy will be used for publication in the JOURNAL.
4. The style and abbreviations of the JOURNAL OF DAIRY SCIENCE must be used. Please refer to abstracts in the June, 1961, JOURNAL for guidance.
5. Only initials of authors should be used, except in unusual cases where it may be necessary to use the complete name.
6. When more than one author is listed, indi-

cate who will present the paper by an asterisk after his name.

7. The title should indicate clearly the nature of the research. It should not be repeated again in the text. The abstract should include, insofar as possible, the design and major results of the investigation. Only complete research should be reported. Brief, essential statistics will make the data more meaningful.
8. The following form with no caps for the title is correct: Utilization of carbohydrates posterior to the rumen-reticulum of the bovine. J. T. Huber and N. L. Jacobson, Iowa State University.
9. If the author lists an address for an experiment station other than the University, such as a USDA research branch or a commercial company, the complete address should be provided, as in the following example: A study of dye reduction methods as platform tests for the detection of antibiotics. Burdet Heinemann, Producers Creamery Co., Springfield, Missouri.
10. All symposium papers should be typed double-spaced and organized according to the style used in the JOURNAL OF DAIRY SCIENCE. The author should send the first copy direct to the JOURNAL Editor, E. O. Herreid, before or immediately following the Annual Meeting.

GRADUATE STUDENT PRESENTATION CONTEST

This contest will be conducted in both the Production and Manufacturing Sections. Each institution is entitled to enter one participant in each contest. They must be student affiliate members. Complete rules for the contest are being sent to department heads. Those wishing to enter the contest must submit copies of their abstract to the Section Officers as outlined above. They should also include a letter indicating their desire to enter the contest. This letter should also be signed by the major Professor and the Department Head. A carbon copy of the letter and four additional copies of the abstract should be mailed to the contest representative.

For Production Section Award: Louis Boyd, Dairy Department, University of Tennessee, Knoxville

For Manufacturing Section Award: A. V. Moore, Department of Dairy Science, Texas A&M College, College Station

Names and addresses of officers of sections to whom titles and abstracts should be sent are:

EXTENSION SECTION

Chairman: W. R. Van Sant, Department of Dairy Science, University of Arizona, Tucson.
Vice-Chairman: C. D. McGrew, Department of Dairy Science, Ohio State University, Columbus 10.
Secretary: D. E. Voelker, Department of Animal Husbandry, Iowa State University, Ames.

PRODUCTION SECTION

Chairman: J. C. Thompson, Ralston Purina Co., St. Louis, Missouri.
Vice-Chairman: L. H. Schultz, Department of Dairy Husbandry, University of Wisconsin, Madison.
Secretary: V. R. Smith, Department of Dairy Science, University of Arizona, Tucson.

MANUFACTURING SECTION

Chairman: M. L. Speck, Department of Food Science and Processing, North Carolina State College, Raleigh.
Vice-Chairman: D. M. Graham, Pet Milk Co., Research and Development Center, Greenville, Illinois.
Secretary: E. L. Thomas, Department of Dairy Industries, University of Minnesota, St. Paul.

(The Dairy Manufacturing Extension Section is a subsection of the Manufacturing Section. W. S. Arbuckle, Department of Dairy Husbandry, University of Maryland, College Park, is chairman, and A. L. Rippen, Department of Food Science, Michigan State University, East Lansing, is secretary. All abstracts for papers in this subsection should be submitted through the regular channels of the Manufacturing Section, but should be identified for presentation at the Dairy Manufacturing Extension Section meeting and a courtesy copy sent to W. S. Arbuckle.)

STATUS OF THE JOURNAL OF DAIRY SCIENCE FOR 1960[1]

E. O. HERREID, Editor-in-Chief
Department of Food Technology, University of Illinois, Urbana

During 1960, 252 papers were published in Volume 43, as compared to 273 in Volume 42 in 1959. There were 198 research papers and technical notes in 1960 and 232 for both sections in 1959. Deducting the interpretive summaries (136 pp.) in Volume 43, comparable total pages for 1959 and 1960 were 2,031 and 1,768, respectively (Table 1). Consolidation

[1] Editor-in-Chief's report to the Executive Board at the 56th Annual Meeting of the American Dairy Science Association, University of Wisconsin, Madison, 1961.

TABLE 1

Distribution of publications in Volume 43 of the JOURNAL OF DAIRY SCIENCE for 1960

Month	Research Papers		Technical Notes		Our Industry Today		Review Papers	Symposia			Our Association		Book Reviews
	M[a]	P[b]	M	P	M	M-P[c]		M	P	M-P[c]	Papers[d]	Abstracts	
						(No.)							
January	4	4	3	2	2		1	1		2	4		
February	7	9	1	1							1	8	3
March	5	6	1			1					1	59	1
April	4	7	2	2		1					1		
May	5	6	1		1	1			2		3		1
June	3	10										243	
July	6	7	2	6							1		2
August	4	11	2								1		1
September	6	12		3				2	2		2		4
October	6	10	1	2				1	2	1	4		1
November	2	9		4	2	1	1			2	3		
December	10	8	1	3	4						3	23	
	62	99	14	23	9	4	2	4	6	5	24	333	13
	161		37		13		2	15			357		13
Total	598												

[a] Manufactures.
[b] Production.
[c] Apply to both Manufactures and Production.
[d] Includes announcements of meetings, committee reports, programs, etc.

of papers submitted by authors in series, more severe trimming of papers, and fewer review papers accounted for fewer pages and titles in Volume 43 as compared to Volume 42. However, the first six issues of Volume 44 contained 1,212 pages, the largest number ever published in the JOURNAL for a comparable period.

JOURNAL TYPOGRAPHY

The format of the JOURNAL is almost stabilized. Changes planned three years ago have been made and will be finalized with Volume 45. Italicized titles for author affiliations, figures, and tables have been eliminated and Roman characters substituted. More appropriate type for section headings and more legible type for the Table of Contents have been installed. These changes were recommended by trained journalists, who were consulted by the Editor.

SPECIAL ACTIVITIES

Publication of supplement. In addition to regular numbers of the JOURNAL for 1960, a supplement was published in April for the papers presented in 1959 at the Fourth Biennial Symposium on Animal Reproduction, held at the University of Illinois. They were excellent contributions to the literature. However, it became evident, when the index for Volume 43 was being prepared, that the publication of isolated supplements or monographs is not a good practice, because it is difficult to index the subjects so that they can be easily retrieved. Furthermore, this supplement confused some members, subscribers, and librarians, as they assumed it was part of Volume 43. For these reasons, symposia which are not functions of the American Dairy Science Association, but contain material appropriate for the JOURNAL, should not be made supplements or monographs but be published as research or review papers in consecutive numbers of the JOURNAL. Reprints can then be supplied under one cover, appropriate for the symposium.

Interpretive summaries. These summaries of research papers, which were published in Volume 43, have been removed from the JOURNAL and now appear in a quarterly publication, the A.D.S.A. Review. The editor obtains these summaries from the authors, edits them, and if necessary returns them to the authors for further revisions. They are finally sent to H. F. Judkins, who assembles and sends them to the Garrard Press.

Review papers. Fourteen competent authorities have agreed to write review papers on timely topics, tentatively scheduled for publication during the next 18 to 24 months.

FOREIGN CONTRIBUTORS

Papers have been received from scientists in Czechoslovakia, Denmark, Italy, Switzerland, and Yugoslavia. Their research is good. They have difficulty in organizing and writing papers

in our language. Their manuscripts were returned with suggestions on how they might be improved for publication.

Inquiries about requirements for membership in the Association and subscriptions for the JOURNAL have been received from seven foreign countries. It was stated in the Editor's report for 1959-60 [2] that the JOURNAL OF DAIRY SCIENCE had only begun to make an impression in countries without a scientific publication. The opportunities to expand our JOURNAL's influence abroad are tremendous. Will the Association take advantage of this opportunity to advance Dairy Science and research abroad? This will take initiative and imagination. Many departments in this country have former students and associates who are members of staffs in universities, experiment stations, and research laboratories in foreign countries. These people are the logical ones to expand the influence and prestige of the JOURNAL and the Association abroad. To a limited number of acquaintances and members abroad, the Editor has suggested that they invite eligible persons to membership in our Association.

Increased growth of the JOURNAL for the sixties is inevitable, provided we want it to grow. It is still a sleeping giant in industry in this country and, as yet, has only begun to make an impact abroad.

QUALITY OF PAPERS

Papers submitted for publication have improved in writing and in research quality during the past three years, but more can be accomplished. About 95% of all papers submitted are returned to the authors for minor to major revisions. Authors frequently return papers, revised according to the recommendations of the Editorial Staff, but they still do not conform in every detail to our JOURNAL style. It is expensive to have such papers standardized uniformly for the printer after they have been accepted for publication. A most time-consuming task is to have reference lists revised that are not properly alphabetized and abbreviated, especially if this is discovered after the papers have been accepted for publication. Sometimes these errors are found while papers are in the reviewing circuit—then the authors are requested to correct them.

It would be unfair to blame authors for all of the nonconformity that appears in their papers, because the paper, Information for Authors,[3] published in 1954, does not have all of the latest and standardized abbreviations, nomenclatures, and terminologies used in papers on applied nuclear physics, chemistry, genetics, and statistics. The result is that authors have used their own variable devices.

[2] J. Dairy Sci., 43: 1706. 1960.

[3] This was written by Dr. P. H. Tracy and published in the J. Dairy Sci., 37: 103. 1954. It was very helpful and adequate at that time.

The abstracts for the Annual Meeting and for the Eastern and Southern Divisional Meetings have been improved, greatly, during the past three years. The abstracts for 1961 were the best ones ever published in the JOURNAL.

It has been very evident that departments and research centers, using local reviewing committees, are submitting superior papers for publication in the JOURNAL. It is sound economies for authors to prepare well-written, concise papers because it costs $25 for one page, or 60 cents for one line, or 6 cents for every word printed in the JOURNAL.

The Editorial Board has been very helpful in suggesting how research papers and those for other sections of the JOURNAL can be revised and improved for publication. The present load for this board is a heavy one. For example, Volume 1 of the JOURNAL contained less than 60 papers,[4] shared for approval by an Editorial Board of 18 to 21 members. Compare this reviewing load for Volume 43, which contained 252 papers, shared by 18 members of the Editorial Board.

GUIDE FOR AUTHORS

The Style Manual for Biological Journals [5] came off the press in late 1960. This manual is the culmination of collaborative work by a dedicated committee, of which J. R. Porter of Iowa State University, Iowa City, is Chairman. Drafts of this manual were read by 100 editors and publishers before it was printed.

This manual specifies accepted and standard abbreviations for bacteriological and chemical terms, symbols, and mathematical formulae and other essential details that will make it possible to prepare, edit, and print manuscripts more efficiently. A section is devoted to writing manuscripts and one to the preparation of the final copy for publication. It is a monumental contribution.

In the development of this style manual the committee assembled from editors of biological journals their various instructions to contributors, such as the paper, Information for Authors.[3] In addition, editors submitted lists of abbreviations, terminology, and symbols which were the outgrowth of more recent applications to nuclear physics, chemistry, and statistics used in their journals. The committee then sorted and assembled all suggestions, which resulted in the publication of the style manual. Obviously, this manual does not define terminology peculiar to every specialized area of biology. For example, there are terms in Dairy Science which need to be standardized with respect to abbreviations and capitalizations. This would require about one page in the JOURNAL.

[4] Some were reports.

[5] Prepared by the Committee on Form and Style of the Conference of Biological Editors. Available from the American Institute of Biological Sciences, 2000 P Street, N. W., Washington 6. D. C.

Since the arrival of the style manual last year, some members of the Editorial Board and 14 other members in the Association have, in unsolicited letters, urged the use of this manual as a guide for the JOURNAL. For this reason, the Editor has held in abeyance a revised version of the paper, Information for Authors, pending a meeting with the Editorial Board [6] at the 1961 Annual Meeting, to decide the best course to follow. There are two choices: use the style manual, amended to include terminology in Dairy Science that needs standardization; or publish a complete revision of the paper, Information for Authors.

CHANGES IN THE EDITORIAL BOARD

There were two resignations from the Editorial Board: Professor F. J. Doan resigned after having served for 10 years. During this time his sound judgment, unimpeachable integrity, patient and careful evaluation of papers, and vision in maintaining a JOURNAL of high quality, have been a tower of strength to three different editors. Obviously, his qualities and experiences can not be fully replaced, but fortunately his counsel is still available as a member of the Journal Management Committee.

Dr. L. M. Smith resigned to accept a Fulbright Grant for study abroad. During the two years he served on the Editorial Board, his prompt, constructive, and courageous evaluation of papers contributed much to improving the quality of the JOURNAL.

Dr. J. P. Mixner resigned because of the time-consuming duties of his new position as Head of the Department of Dairy Science at Rutgers University. His outstanding research in physiology and endocrinology made him eminently qualified to evaluate papers in these areas. It is a pleasure to report that Dr. E. P. Reineke of Michigan State University has consented to again become a member of the Board.

Dr. C. A. Zittle, a distinguished member of the staff at the Eastern Regional Laboratory of the United States Department of Agriculture in Philadelphia, Pennsylvania, has consented to become a member of the Board and assist in evaluating papers in the area of physical chemistry.

Dr. Robert Jenness has been a devoted, faithful, and conscientious member of the Board for nine years. He has been awarded a Fulbright Scholarship to conduct research on milk proteins at the Netherlands Institute for Dairy Research at Ede, beginning September 1.

It is a pleasure to welcome to the Editorial Board again Dr. E. L. Jack, who replaced Dr. L. M. Smith, and Dr. C. K. Johns, for his first term as the replacement for Professor F. J. Doan.

Because of the increased numbers of very technical papers in genetics and breeding, it was necessary to get relief in this area. The Board was fortunate to again obtain Dr. R. W. Touchberry, whose return is welcomed after an absence of two years.

To relieve members of the Editorial Board in chemistry and technology, Dr. U. S. Ashworth consented to assist in these areas. We are happy that he accepted this responsibility, as the JOURNAL will benefit from his experience as a writer and researcher.

ACKNOWLEDGMENTS

The helpful counsel of Professor F. J. Doan and the members of his Journal Management Committee is sincerely appreciated.

To the Editorial Board, appreciation is expressed for indispensable help in constantly elevating the quality and maintaining the integrity of the JOURNAL.

The Editor-in-Chief is indebted, greatly, to Dr. Glenn Hanson and Dr. Paul Fisher of the Schools of Journalism at the Universities of Illinois and Missouri, respectively, for their generous help in improving the appearance, format, and organization of the JOURNAL.

The generous and efficient cooperation from the program committee was most helpful in achieving publication deadlines for the program and abstracts for the Annual Meeting. Without this help the Editor's position would have been untenable. Thanks and appreciation go to Dr. G. W. Trimberger, General Chairman, Mr. B. Heinemann, Chairman of the Manufacturing Section, Dr. S. D. Musgrave, Chairman of the Production Section, Dr. M. E. Senger, Chairman of the Extension Section, and Dr. R. P. Niedermeier, who, with these alert chairmen, assembled the most nearly perfect program that we have ever had for publication.

Finally, R. J. Garrard, President of the Garrard Press, and his associates C. L. Hopper and Lois Hough, have cooperated in every way to maintain editorial accuracy and integrity. It is with regret that we lost the services of Mr. Hopper, who for the past two years has taken an interest in the JOURNAL far beyond the call of duty. Mr. Claude Cruse has assumed Mr. Hopper's duties and we can expect the same efficient liaison and mutual cooperation between the Editor's office and the Garrard Press.

[6] The Style Manual for Biological Editors was recommended for adoption by the Editorial Board and was approved by the Journal Management Committee and by the Executive Board at the 56th Annual Meeting, University of Wisconsin, Madison, 1961.

PRICE SCHEDULE FOR REPRINTS OF PAPERS THAT APPEAR IN THE JOURNAL OF DAIRY SCIENCE

H. F. JUDKINS, Secretary-Treasurer
32 Ridgeway Circle, White Plains, New York

The Executive Board, at the time of the Annual Meeting of the American Dairy Science Association at the University of Wisconsin, increased the price of reprints 25%, effective July 1, 1961. The new reprint schedule follows:

No. of reprints	Number of pages								
	2	4	8	12	16	20	24	28	32
	(Cost in dollars)								
50	17.50	20.00	36.25	51.25	67.50	78.75	97.50	115.00	125.00
100	20.00	22.50	41.25	61.25	77.50	92.50	112.50	132.50	145.00
200	22.50	28.75	51.25	76.25	97.50	117.50	143.75	162.75	185.00
300	28.00	33.75	62.50	91.25	117.50	143.75	173.75	205.00	226.25
400	30.00	40.00	72.50	107.50	137.50	170.00	205.00	241.25	266.25
500	33.75	45.00	83.75	122.50	157.50	195.00	236.25	277.25	306.25
600	37.50	51.25	93.75	137.50	177.50	221.25	266.25	313.75	346.25
700	41.25	56.25	105.00	153.75	197.50	246.25	297.50	350.00	387.50
800	45.00	62.50	115.00	168.75	218.75	272.50	328.75	386.25	427.50
900	48.75	67.50	126.25	185.00	238.75	298.75	358.75	422.50	467.50
1,000	57.25	73.75	136.25	200.00	258.75	323.75	390.00	458.75	507.50

If covers for reprints are desired, the cost of 50 covers will be $12.18, and for each additional 100 covers, the cost will be $8.75. Back copies of the JOURNAL will cost $2 each.

The reprints are made from standing type within 30 days after the papers appear in the JOURNAL. Requests for a few reprints of a paper should be sent to the authors, whose names and addresses appear with the title. The Secretary and the Editor's office do not keep supplies of the various reprints. Orders for large numbers of reprints should be sent to The Garrard Press, 510 North Hickory Street, Champaign, Illinois. These orders must be received within 30 days after the papers are published in the JOURNAL; otherwise, the type will have been destroyed.

In case the original type has been destroyed, it is possible to supply reprints by a special photographic process, and their cost will be 50% more than the regular ones. For example, 100 reprints of 32 pages will cost $217.50.

It is hoped that the publication of this reprint schedule will make it easier for interested people to obtain reprints in any number desired and, at the same time, aid in disseminating useful information to the dairy and related industries.

The JOURNAL OF DAIRY SCIENCE is copyrighted. Reproduction of complete papers from this JOURNAL by any organization is not permitted. The reproduction of graphs, tables, and illustrations for books and other periodicals may be authorized by the Editor-in-Chief.

DIFCO

BRUCELLA

Isolation, Cultivation and Differentiation

BACTO-TRYPTOSE

is the peptone of choice in the preparation of both liquid and solid media for culturing *Brucella abortus, melitensis* and *suis* and supplies the nutriments required by these organisms for rapid and abundant growth.

BACTO-TRYPTOSE BROTH

is a complete liquid medium for culturing the *Brucella* and is especially adapted to the isolation techniques recommended by Huddleson and Castaneda.

BACTO-TRYPTOSE AGAR

supersedes media previously employed for the isolation and cultivation of the *Brucella*. This medium serves ideally for the primary or secondary isolation of *Brucella*, for the differentiation of species and for vaccine or antigen production. It is also recommended for use as the solid phase in the Castaneda technique.

THE DIFCO MANUAL, *NINTH EDITION,*
including descriptions of these media and their use, is available on request.

Specify DIFCO — the trade name of the pioneers in the research and development of Bacto-Peptone and Dehydrated Culture Media

DIFCO LABORATORIES
DETROIT 1, MICHIGAN